TRANSPORT OF CARNITINE IN RBE4 CELLS - AN *in vitro* MODEL OF BLOOD-BRAIN BARRIER.

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SUMMARY

Accumulation of carnitine was studied in immortalized rat brain capillary endothelial cells RBE4, as an *in vitro* model of blood-brain barrier. Both uptake and efflux phenomena were found independent on [Na^+] gradient. A quick acylation of carnitine in RBE4 cells was observed, especially leading to formation of long-chain acylcarnitines (46%), while 38% of the accumulated compound was found in the form of free carnitine. Exposure of basolateral membrane to carnitine-free medium induced a quick efflux, leaving, however, about 50% of accumulated carnitine inside the cells. The process of accumulation was found to be sensitive to butyrobetaine and cysteine, but not to choline or Hemicholinium-3, pointing to sterical demands of a transporter responsible for carnitine uptake.

KEY WORDS: carnitine, blood-brain barrier, membrane transport

INTRODUCTION

The accumulation of any compound in brain depends on the selectivity of the brain capillary endothelial cells connected by tight junctions and providing a permeability barrier between blood and brain fluids, the so called blood-brain barrier. Selective permeability of this barrier is created by expression of specific genes, e.g. GLUT1 of glucose transporter (1). Several transporting systems have been described to be present in the blood-brain barrier: for glucose and other hexoses (1-3), for neutral and basic (4), as well as acidic amino acids (5), for

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monocarboxylic acids (3, 6, 7), for choline (8, 9) and purine bases (10). There is also a report on the presence of a transport system for vitamins (11). In addition, specific transporters for sodium have been described in brain capillaries (12).

One of the substances which accumulate in brain is carnitine (4-N-trimethylammonium-3-hydroxybutyric acid), known to be involved in peripheral tissues in transfer of acyl compounds from the cytosol to the mitochondrial matrix for further metabolism (13). It has been postulated that in neurones, where the level of fatty acid ß-oxidation is relatively low (14) carnitine can fulfill a different physiological role, namely export of acetyl moieties from mitochondria to cytosol for acetylcholine synthesis (15-17). The amount of accumulated carnitine was shown to be different in various regions of canine brain, the highest being reported for cerebellar cortex (18), while in rat brain the hypothalamus turned out to have the highest content of free carnitine and its acyl derivatives (19). Since hypothalamus has an easy access to many substances circulating in blood through fenestrations, this observation may point to some limitations of carnitine crossing through the blood-brain barrier, at least in rat. Therefore this study was focused on characterization of the carnitine transport through rat blood-brain barrier using a model system.

Till now, primary cultures of brain capillary endothelial cells were considered the best *in vitro* model for studies on the blood-brain barrier (20, 21). Recently, an alternative model was proposed, namely immortalized brain microvessel endothelial cells. The established RBE4 cell line derived from rat brain (22) has been used in this study.

MATERIALS AND METHODS

Materials: RBE4 cells from rat brain microvessel endothelial cells were immortalized by transfection with the plasmid pE1A-*neo*, containing the adenovirus E1A gene, as described in (22). Tissue plastics were from Corning. Minimum essential medium (Alpha medium), Ham's F-10 nutrient mixture, fetal bovine serum, L-glutamine, gentamycin, geneticin and collagen G were provided by Gibco, basic fibroblast growth factor by Boehringer. L-[Methyl-^{3}H]carnitine and [methyl-^{14}C]thymidine were purchased from Amersham. L-acylcarnitines were delivered by Serva, L-carnitine was provided by Fluka. Butyrobetaine ((3-carboxypropyl)trimethyl-ammonium) was synthesized by methylation of GABA by Dr. J. Boksa in the Institute of Pharmacology, Cracow, Poland. All other reagents were from Sigma. Aluminium silica gel 60 (sheets) was from Merck. Ultima Gold (Packard) was used as a scintillation cocktail.

Cell culture: RBE4 cells were grown on collagen-coated dishes in Alpha medium/Ham's F-10 (1:1) supplemented with 2 mM glutamine, 10% heat-inactivated fetal bovine serum, 1 ng/ml basic fibroblast growth factor, 0.3 mg/ml geneticin, 10 µg/ml gentamicin, L-[methyl-^{14}C]thymidine (3.5 nCi/ml) pH 7.4. Cells were grown to confluence at 37°C in a humid atmosphere of 5% CO_2.

Accumulation of carnitine: Culture medium was removed and cells were washed with phosphate-buffered saline (PBS). The cells were subsequently incubated at 37°C as a monolayer in PBS supplemented with 5 mM glucose. The reaction was started by addition of 50 µM L-[methyl-^{14}C]carnitine (15 Ci/mol) and terminated by removal of the incubation medium. Subsequently, the monolayer was washed three times with ice cold PBS, the cells were collected in PBS with a cell scraper, and spun down at 5,800g for 10 min at 4°C. For the experiments performed with cells in suspension, the cellular monolayer was collected with a cell scraper in PBS with 5 mM glucose before incubation, and the reaction was terminated by spinning down at 5,800g for 10 min at 4°C, followed by three washes by centrifugation. For

measurement of the total carnitine and thymidine accumulation, cells were dissolved by incubating overnight in 0.1 M NaOH, 2% Na_2CO_3, 1% sodium dodecyl sulfate at 40°C and taken for double label radioactivity counting. The results were calculated as the amount of carnitine referred to the thymidine incorporation, since the protein content could not be measured due to collagen covering the culture dishes. The unspecific binding was subtracted as the "zero time uptake". For estimation of carnitine and its derivatives, the reaction was performed in the same way, the cellular pellet after termination of the reaction was treated with 10% trichloroacetic acid and the further procedure of carnitine and its acyl derivatives separation was performed by thin layer chromatography according to (23). For efflux measurements, the cells were incubated in the presence of 50 μM L-[methyl-^{14}C]carnitine (Ci/mol) either as a monolayer or as suspension. After preloading the RBE4 cells for 2.5 h with L-[methyl-^{14}C]carnitine the medium was removed either directly from monolayer or from cell suspension after centrifugation at 5,800g for 10 min. Cells were next incubated for indicated times in PBS with 5 mM glucose (without carnitine). In case of cell monolayer the reaction was terminated as described for carnitine accumulation. The cells in suspension were spun down at 5,800g for 10 min and washed three times with PBS by centrifugation performed under the same conditions. The amount of labeled carnitine left in the cells was estimated by radioactivity counting.

RESULTS

In order to cross plasma membrane, an ionized, low molecular weight compound can be transported either downhill its concentration gradient by a facilitated diffusion, or in an uphill transport when its movement across the membrane is coupled to a process in which other molecules move down their free energy gradient (24). In case of plasma membrane many transporters operate using Na^+ concentration gradient and, in general, the Na^+/solute symporters have been classified into at least 11 families on the basis of their sequence and functional similarities (25). Therefore, when studying the carnitine transport in RBE4 cells, it seemed crucial to clarify whether carnitine accumulation, if any, is coupled with [Na^+] gradient and whether some of the known transporters could be involved in this process.

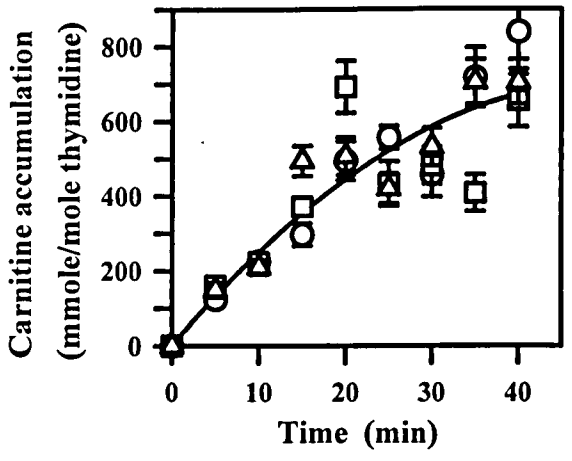

Fig.1. Accumulation of carnitine in the monolayer of RBE4 cells.
Cells were incubated in the presence of 50 μM carnitine, as described in Materials and Methods, without any additions (circles) and with either 1 mM furosemide, 0.1 mM amiloride (squares) or after 30 min preincubation with 0.5 mM ouabain (triangles). The results are means±SD from 3 independent experiments.

As presented in Fig.1, RBE4 cells are able to accumulate carnitine. The amount of accumulated compound, measured as a function of time, resulted in a curved graph, which could be fitted (with use of a nonlinear regression) to the first-order rate equation, with $k = 0.035 \pm 0.02$ min^{-1} as the velocity constant.

In order to check a possible involvement of sodium concentration gradient as a driving force for carnitine uptake, compounds affecting Na transport through the plasma membrane were added. Amiloride, the sodium channel inhibitor and furosemide, the inhibitor of Na^+-K^+-$2Cl^-$-cotransporter, at concentrations affecting sodium transport in brain (12), were found without effect on carnitine accumulation. A well established inhibitor of Na,K-ATPase, ouabain, (26) did not reveal any effect either (Fig.1). This enzyme, however, is known to be localized at the basolateral membrane (27) which in cells grown on collagen would be attached to the culture dish. Therefore, a possible influence of all inhibitors, including dimethylamiloride -

TABLE I. Effect of Na+ transport inhibitors on the accumulation of carnitine by RBE4 cells.

ADDITION	RELATIVE CARNITINE ACCUMULATION
None	100
Furosemide (1 mM) + amiloride (0.1 mM) + dimethylamiloride) (0.02 mM)	96 ± 8
Ouabain (0.5 mM)	103 ± 9
All inhibitors	86 ± 8

The cells were incubated in suspension with 50 μM carnitine and the uptake was measured as given in Materials and Methods. The values represent the relative values of initial velocities (6 measurements) ± SD.

which inhibits Na/H exchanger (28), was checked as well, when the cells were incubated in suspension, thus making Na,K-ATPase accessible for its inhibitor. As presented in Table I, there was no effect observed in the presence of any of the compounds added, pointing to the fact

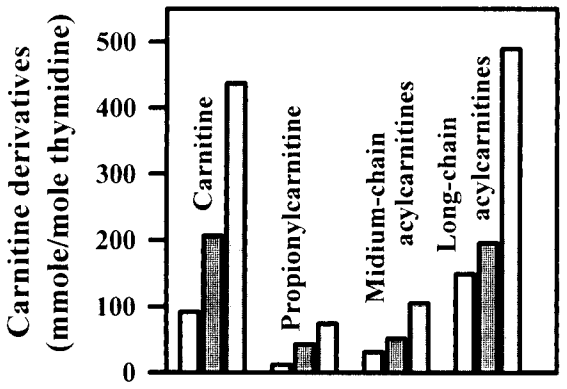

Fig.2. Distribution of carnitine and its derivatives in RBE4 cells. The cells were incubated with 50 μM carnitine for 5, 20 and 60 min, shown by bars arranged in each group from the left to the right, respectively. Separation of carnitine and its derivatives was performed, as described in Materials and Methods.

that carnitine accumulation in RBE4 cells is not dependent on sodium concentration gradient.

Carnitine, when accumulated in the cells, has been shown to become a substrate of carnitine acyltransferases, enzymes specific toward acylCoA derivatives of various chain length (13). Analysis of acylcarnitine content in RBE4 cells allowed estimation of the content of free carnitine and its derivatives. The amount of all compounds was observed to increase in time (Fig.2) and long-chain acylcarnitines were found to account for 46±6% of the total accumulation. The relative content of other derivatives was very low, reaching 6±1% for propionylcarnitine and 10±1.5% for medium-chain acylcarnitines. Free carnitine was detected to represent 38±4% of the total accumulation. Such a distribution, being different from the one reported for neurones (29), would point to a substantial difference in carnitine acyltransferases' activities between endothelial and neural cells.

A compound able to cross the blood-brain barrier would not only accumulate in endothelial cells, but subsequently cross the basolateral membrane in order to reach brain fluid. When carnitine was removed from the external medium, no loss of intracellular carnitine was observed in cell monolayer (Fig.3). The same experiment performed with cells in suspension resulted, however, in a very quick efflux, the initial velocity of which could not be measured. Nevertheless, approximately half of accumulated carnitine was found always

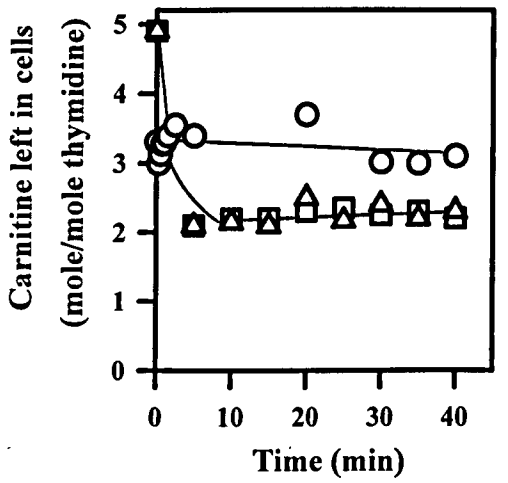

Fig.3. Efflux of carnitine from RBE4 cells. The cells were incubated in the presence of 50 μM carnitine for 2 h, either in monolayer (circles) or in suspension (squares, triangles). Carnitine was then removed and the cells were incubated for indicated time in PBS with 5 mM glucose either without any aditions (circles and squares) or with amiloride, furosemide, ouabain (triangles) at concentrations given in the Legend to Fig.1. The reaction was terminated as described in Materials and Methods.

remaining in the cells. This process of carnitine efflux has to be described as independent on sodium concentration gradient, since it was not affected by inhibitors of Na transporting systems (Fig.3). Differences observed in efflux phenomena in monolayer and in suspension could indicate that endothelial cells do not loose carnitine easily through the apical membrane (monolayer), since the efflux was much faster, when the whole plasma membrane was exposed to the carnitine -free medium. Thus this efflux would probably occur through

the basolateral membrane. It has to be emphasized that accumulation of carnitine after 2.5 h of preloading, before starting the efflux measurements, was found much higher in suspended cells than in the monolayer (Fig.3, time 0). This could point to a possibility that basolateral membrane contains an additional carnitine-accumulation system, not exposed in polarized cells attached by their basolateral side to collagen-coated dishes.

When looking for a system which might be responsible for carnitine binding and transport in RBE4 cells, one may focus on a possible involvement of the polar, functional groups of carnitine. Choline, although structurally very similar to carnitine, was found,

TABLE II. Effect of different compounds on accumulation of carnitine in RBE4 cells.

ADDITION	CONCENTRATION	% OF CONTROL UPTAKE
Choline	1 mM	102 ± 6 (10)
Choline	10 μM	101 ± 12 (8)
Hemicholinium-3	2 μM	96 ± 11 (7)
Betaine	1 mM	111 ± 10 (9)
Butyrobetaine	1 mM	71 ± 9 (7)
Cysteine	1 mM	88 ± 10 (7)
Cysteine	5 mM	71 ± 15 (5)
Cysteine	10 mM	43 ± 15 (5)

The RBE4 cells were incubated up to 60 min with 50 μM carnitine in the presence of different compounds added simultaneously. The reaction was terminated, as specified in Materials and Methods. The amount of initial velocity measurements is given in parentheses.

however, without any effect on carnitine accumulation (TABLE II), even at concentrations exceeding saturation of the low affinity system (30). Carnitine uptake was not changed either in the presence of Hemicholinium-3, an inhibitor of the system transporting choline with a high afiinity (31). Betaines, i.e. compounds lacking a hydroxyl group in comparison with carnitine, were found to have various effects: betaine itself did not influence carnitine accumulation, while butyrobetaine inhibited this process by 30%. This points not only to the importance of a carboxyl and trimet'ylammonium groups for carnitine transport, but also to the significance of the chain length between these functional groups. In neurones (29) carnitine uptake was found strongly inhibited by cysteine. Accumulation of carnitine by RBE4 cells was also observed to be sensitive to cysteine in a concentration dependent way (TABLE II). It has to be emphasized, however, that carnitine transport in neurones was found Na-dependent, possibly involving carboxyl and hydroxyl groups (29) and thus different from the transport herewith.

DISCUSSION

Several methods have been applied in studies on transport phenomena through the blood-brain barrier. Classical measurements *in vivo* have been using techniques of either constant infusion or single injection, later developed by Oldendorf (32) into a tissue -sampling-arterial injection technique. These methods are especially useful when, for instance, the effect of aging, the influence of pathological states or nutritional status are explored. Classical studies on the blood-brain barrier *in vitro* used isolated brain capillaries (33-35) and, more recently, primary cultures of capillary endothelial cells (20, 36). *In vitro* systems, which are more pure allow to define more precisely functional and kinetic characteristics of given transporting system, as well as to draw conclusions concerning possible cellular polarity. RBE4 cells, which are not contaminated with other cell types, form an excellent system not only for functional studies of the blood-brain barrier *in vitro*, but also for purification attempts of a specific transporter.

It can be concluded from studies on carnitine transport presented herewith, that the accumulation of this compound does not depend on $[Na^+]$ gradient. Observed fast action of carnitine acyltransferases, removing ~60% of free carnitine from the cytosol, suggests that carnitine could be transported down its concentration gradient by a facilitated diffusion. The transporter responsible for such a process, localized at the apical membrane, proved to have certain structural demands concerning its substrate, namely N-trimethylated and carboxyl groups, separated by four-carbon chain. The observed inhibition by cysteine can not be ascribed to the involvement of ASC (alanine, serine, cysteine specific) transporter, which is known to be Na-dependent and not tolerating N-methylation (37). Thus the effect of cysteine could be rather interpreted as an involvement of SH group(s), but the latter conclusion requires further investigation. Similarly, suggestion that RBE4 basolateral membrane may contain and additional (different?) carnitine-translocating system still needs further studies. Observations concerning a fast efflux through basolateral membrane suggest that brain endothelial cells *in vivo* would be able to transport ~50% of accumulated carnitine to neurones and glial cells. Since the physiological concentration of carnitine in blood (38) is lower than the K_m of its uptake by neurones (29), the system of carnitine accumulation in endothelial cells appears fully able to regulate the accessibility of this compound to neurones.

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