L-Carnitine Transport in Kidney of Normotensive, Wistar–Kyoto Rats: Effect of Chronic L-Carnitine Administration

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Purpose. To examine the effect of long-term administration of L-carnitine on L-carnitine transport in renal brush-border membrane vesicles (BBMVs) from normotensive, Wistar-Kyoto rats.

Methods. Rats (n = 20) were orally administered 0.2 g carnitine/kg body weight per day for a total period of 8 weeks. Kinetic parameters of L-carnitine uptake were calculated by non-linear regression, and the relative abundance of the carnitine transporter, OCTN2, was determined by Western blot analysis.

Results. Initial rates and maximal overshoot levels of Na^+-dependent L-carnitine transport were significantly reduced in BBMVs from L-carnitine-treated rats compared with untreated animals. Similarly, the maximal transport rate (V_{max}) of OCTN2 was lower in treated rats. However, no differences were observed in the Michaelis constant (K_m) or the diffusion constant (K_d) between the two groups of animals. The amount of OCTN2 protein was also decreased in L-carnitine-fed rats, this reduction being similar to that of the V_{max}. These results were accompanied by an increase in the serum levels and also in the renal excretion of both free and esterified carnitine in treated rats, indicating that the long-term administration of L-carnitine leads to increased renal carnitine clearance.

Conclusion. These findings suggest a downregulation of OCTN2 at the renal level, in the presence of high levels of carnitine.

KEY WORDS: L-carnitine transport; OCTN2; kidney; brush-border membrane vesicles; Wistar–Kyoto rats.

INTRODUCTION

L-carnitine (β-hydroxy-γ-(N-trimethylammonium)butyrate) is an essential cofactor for the transport of long-chain fatty acids from the cytosol to mitochondria for subsequent β-oxidation and production of cellular energy. It is found in significant quantities in meat and dairy products and synthesized mainly in the liver and to a lesser extent in kidney, from the amino acids lysine and methionine (1). Therefore, both sources are responsible for the maintenance of carnitine levels in plasma and tissues (2). The kidney has an important role in the homeostatic regulation of carnitine concentration in body fluids. Carnitine is filtered and reabsorbed by more than 95% in the proximal tubule (3), and a small quantity is eliminated in the urine as free carnitine or acylcarnitine (2).

In animals and human tissue samples (4), it has been reported that L-carnitine transport is performed by a family of membrane proteins, namely OCT (organic cation transporters). So far, three members of this family, OCTN1, OCTN2, and OCTN3, have been identified and showed their ability to transport carnitine, but with variable characteristics in terms of affinity and capacity for carnitine transport, as well as sensitivity to inhibitors (5).

In the kidney, L-carnitine transport has been well characterized, and it is primarily mediated by a sodium-dependent carnitine transporter, OCTN2 (6–8), which is located on the luminal side of epithelial cells (9). In addition, it has been published that several mutations of OCTN2 are responsible for the primary carnitine deficiency (4), a syndrome characterized by an increased renal excretion of carnitine, low carnitine plasma and tissue levels, and decreased oxidation of fatty acids (10). This demonstrates the important role of OCTN2 in the renal tubular transport of carnitine, and a similar syndrome has also been described in juvenile visceral steatosis mice in which renal reabsorption of carnitine is lacking (11).

Patients with carnitine deficiency usually develop myopathy, cardiomyopathy, encephalopathy, and a variety of biochemical changes (10,12). Treatment with carnitine is lifesaving in these patients, but high daily doses are needed to override the increased renal excretion of carnitine. Moreover, and despite the important physiologic function of carnitine, the factors regulating carnitine transporters are not well understood. It has been postulated that serum carnitine levels might play a regulatory role in carnitine uptake (13). Interestingly, there is increasing evidence suggesting a beneficial effect for carnitine therapy in a number of cardiovascular disorders, including angina pectoris, acute ischemia, congestive heart failure, hyperlipidemia (14,15), and arterial hypertension (16). Based on the pathophysiological consequences and treatment possibilities of L-carnitine in arterial hypertension, the purpose of the present work was to examine the renal carnitine transport after a long-term treatment with carnitine. We used brush-border membrane vesicles (BBMVs) isolated from kidney cortex of Wistar–Kyoto (WKY) rats, the usual normotensive control for spontaneously hypertensive rats because our interest in renal carnitine absorption originated from our previous investigation into the arterial hypertension field.

MATERIALS AND METHODS

Animals

Normotensive WKY rats aged 3–4 weeks were obtained from Harlan IBERICA, S.A. (Barcelona, Spain). Rats were kept under standard conditions (23 ± 1°C, 12-h light/12-h dark cycles) and were fed a standard pellet diet with free access to tap water. Rats were divided at random into two groups of 20 animals each. In one of the groups, an L-carnitine solution was offered for 8 weeks instead of water. The amount of consumed carnitine was about 0.2 g/kg body weight per day, adjusted from their daily water consumption. At the end of the experimental period, a 24-h collection of urine was performed. Rats were fasted for 18 h before killing, and blood were obtained from cardiac puncture and centrifuged at 3000 × g for 10 min. Urine and serum aliquots were stored at –70°C until assay. Kidneys were quickly removed, weighed, and decapsulated before cutting slices from the renal cortex. All studies were conducted in accordance with the Principles of Laboratory Animal Care (National Institutes of Health Pub-

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lication No. 85-23, revised 1985) and approved by the local standard for protecting animal welfare.

Carnitine Determinations

The levels of free, esterified, and total carnitine in serum and urine were determined by a colorimetric method as previously described (17). Serum was prepared for analysis in 3% perchloric acid (v/v). The perchloric acid-soluble fraction was assayed directly for free carnitine, and after alkaline hydrolysis for total acid-soluble carnitine (representing the sum of free carnitine and short-chain acylcarnitine esters). The levels of short-chain acylcarnitine esters were obtained by subtraction (total acid-soluble minus free carnitine content). The content of long-chain acylcarnitine esters was determined in the perchloric acid pellets. Total carnitine content refers to the sum of long-chain acylcarnitine content and total acid-soluble carnitine content. Esterified carnitine refers to the sum of long-chain and short-chain acylcarnitine esters. Urine was assayed directly for free carnitine and total carnitine.

Renal clearance of free carnitine was calculated as the ratio urinary excretion rate / serum concentration. Serum and urinary creatinine were measured as previously described (18), and renal clearance of creatinine (reflecting glomerular filtration, GF) was calculated as the ratio urinary excretion rate / serum concentration. Fractional excretion (FE) of free carnitine, that is, the percentage of filtered free carnitine excreted in the urine, was calculated as follows:

$$FE\ (\%) = [\text{Urinary excretion } (\mu\text{mol/24 h}) \times 100]/ [\text{Serum concentration } (\mu\text{mol/L}) \times GF],$$

where *GF* represents the glomerular filtration as determined from the creatinine clearance (L/24 h).

Preparation of BBMVs

Renal cortical BBMVs were prepared from WKY and L-carnitine-treated WKY rats by a $MgCl_2$ precipitation method, as previously described in detail (19). The final pellets containing purified BBMVs were resuspended in the buffers used in transport experiments, whose compositions are reported in uptake studies, and homogenized with 25- and 29-gauge needles. The vesicles were frozen and stored in liquid nitrogen until use (for a period no longer than 15 days). For each preparation of BBMVs, renal cortex from two rats was used. Preparation of BBMVs was always run in parallel on the same day from animals of both experimental groups.

Protein and Enzyme Activity Determinations

Protein determination was carried out by the method of Bradford (20) using a bovine gamma-globulin as a standard. The membrane preparation process was evaluated by measuring the specific activities of marker enzymes. Alkaline phosphatase was used as the marker enzyme for brush-border membrane vesicles and estimated by the method of Bretaudiere *et al.* (21). The basolateral membrane marker enzyme, Na^+-K^+-ATPase, was measured according to Colas and Maroux (22). Succinate dehydrogenase, as marker of mitochondria, and acid phosphatase, as marker of lysosomes, were also determined as previously described (23,24). All enzyme activities were measured at 37°C.

Determination of the Transport Capability and Intravesicular Space

The transport of Na^+-dependent D-glucose was measured to control the functional integrity of the isolated BBMVs and determine the intravesicular volume. Five to ten microliters of BBMVs, previously resuspended in a loading buffer (in mmol/L: 300 mannitol, 0.1 $MgSO_4$ and 20 HEPES/Tris, pH 7.4), were combined with 100 μL of an incubation medium (in mmol/L: 100 mannitol, 0.1 $MgSO_4$, 20 HEPES/Tris, pH 7.4) containing 0.1 mmol/L D-(^{14}C)glucose and 100 mmol/L of either NaCl or choline chloride. The functional integrity and the intravesicular space were calculated at 15 s and at equilibrium time (30 min), respectively, at 37°C and using the rapid filtration technique described elsewhere (19).

L-Carnitine Transport

For the uptake of L-carnitine (measured at 37°C), BBMVs were loaded in a medium containing, in mmol/L, 300 mannitol, 0.1 $MgSO_4$, and 20 HEPES/Tris, pH 7.4 except for the studies in which the influence of the membrane potential on the activity of L-carnitine transporter was determined (100 mannitol, 100 KCl, 0.1 $MgSO_4$, and 20 HEPES/Tris, pH 7.4). The incubation medium (in mmol/L: 100 mannitol, 0.1 $MgSO_4$, and 20 HEPES/Tris, pH 7.4) contained 50 μmol/L L-(^{3}H)carnitine (0.2 μCi per assay) and 100 mmol/L of either NaCl or choline chloride. For transmembrane electrical potential studies, 45 μmol/L valinomycin was added to the incubation medium. Na^+-dependent L-carnitine uptake was determined by subtracting the uptake in the presence of a Na^+ gradient from that with no Na^+ gradient (choline gradient). Inhibition studies (50 μmol/L L-carnitine) were conducted in the presence of various carnitine analogues, L-acetylcarnitine, L-propionylcarnitine, and L-palmitoylcarnitine (1 and 3 mmol/L), and also in the presence of D-glucose (100 μmol/L). For the kinetic analysis, L-carnitine concentrations were 1, 2, 5, 10, 25, 50, and 100 μM.

Intra- and extravesicular media were isotonic (320 mosmol/L), except for the experiments in which the effect of increasing osmolarity on substrate uptake was determined. In these experiments, mannitol was added to the incubation medium to give the indicated osmolarity.

Kinetic Analysis

Total L-carnitine fluxes were analyzed by nonlinear regression using the Enzfitter program (Biosoft, Cambridge, UK). Because errors associated with experimental fluxes were roughly proportional to their values, it was considered appropriate to apply a proportional weighing to the data. Kinetic parameters were calculated considering a model equation comprised of one saturable, Michaelis-Menten component plus a linear, nonspecific component.

Western Blot Analysis of OCTN2

BBMVs (equivalent to 100 μg protein) from WKY and L-carnitine-treated WKY rats were solubilized in Laemmli sample buffer and resolved by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were then electrotransferred onto nitrocellulose membranes, which were incubated with antisera raised in rabbits against a 17-aa synthesized peptide corresponding to the cytoplasmic C-terminus of

mouse OCTN2 (Alpha Diagnostic Intl. Inc., San Antonio, TX, USA). The anti-OCTN2 antibody was detected by the enhanced chemiluminescence method as previously described (19), and the samples were measured by scanning densitometry. To avoid the possibility of artifacts from manipulation, OCTN2 antibody was stripped off the membranes, which were later incubated with a mouse anti-actin monoclonal antibody according to the protocol described above.

Statistical Analysis

Comparison between different experimental groups was analyzed by the unpaired, two-tailed Student's *t* test, and differences were considered significant at $p < 0.05$.

Materials

All unlabeled reagents were obtained from Sigma Chemical (Madrid, Spain), except for the reagents used to determine protein and for Western blot analysis, which were from Bio-Rad (Barcelona, Spain). D-(U-^{14}C)glucose, L-(^{3}H)carnitine and the enhanced chemiluminiscence reagent were obtained from Amersham International (Madrid, Spain). The membrane filters were obtained from Millipore (Barcelona, Spain).

RESULTS

Serum Concentration and Urinary Excretion of Carnitine

As shown in Table I, the treatment with L-carnitine led to an elevated urinary excretion of free (175-fold), esterified (7-fold), and total (42-fold) carnitine. Similarly, serum levels of free, esterified, and total carnitine were enhanced by factors of 10, 8, and 8, respectively, in L-carnitine-treated WKY rats. Therefore, the higher increase was observed in the levels of free carnitine in both urine and serum. Creatinine clearance (i.e., the glomerular filtration) did not change between both groups of rats. However, the renal clearance of free carnitine was increased in rats treated with carnitine by a factor of approximately 18. The observed increase in the urinary excretion of free carnitine in L-carnitine-treated rats was due to a lower renal free carnitine reabsorption rate (99% vs. 89% for control and treated rats, respectively), as indicated by the normal glomerular filtration but higher fractional excretion of free carnitine in treated rats.

Table I. Serum Concentration and Urinary Excretion of Carnitine in Control and L-Carnitine-Treated (LC) Wistar–Kyoto Rats

Sample	Carnitine form	Control	LC
Serum (μmol/L)	Free	8 ± 0.3	76.1 ± 4.8***
	Esterified	11.5 ± 2.1	85.9 ± 8***
	Total	19.4 ± 2	163 ± 10***
Urine (μmol/24 h)	Free	0.14 ± 0.02	24.5 ± 1.2***
	Esterified	0.54 ± 0.04	4 ± 0.5***
	Total	0.68 ± 0.03	28.5 ± 1.2***
Creatinine clearance (L/24 h)		3 ± 0.2	2.9 ± 0.3
Free carnitine clearance (mL/24 h)		17.2 ± 2.3	321 ± 28***
FE free carnitine (%)[a]		0.6 ± 0.05	11.1 ± 0.3***
RR free carnitine (%)[b]		99 ± 0.5	89 ± 1.5**

Note: Values are means ± SE for 10 animals. **$p < 0.01$ and ***$p < 0.001$ compared with the control group.
[a] FE = fractional excretion.
[b] RR = reabsorption rate.

Purity and Functionality of BBMVs

Table II shows the enzyme specific activities in homogenate and brush-border membranes prepared from kidney cortex of WKY and L-carnitine-treated WKY rats. Alkaline phosphatase was 12-to 13-fold enriched in the final brush-border membranes compared with the starting homogenate, and the recoveries of this enzyme were high enough (34–43%) in both experimental groups. Enrichments and recoveries of Na^+-K^+-ATPase, acid phosphatase, and succinate dehydrogenase were low, indicating very little basolateral, lysosomal, and mitochondrial contamination, respectively.

The transport capability of the BBMVs was assessed by determining D-glucose uptake. The presence of a Na^+ gradient across the vesicles ($Na_o^+ > Na_i^+$) showed a transient accumulation of D-glucose in both experimental groups at 15 s (535 ± 52 vs. 518 ± 62 pmol/mg protein for control and treated rats, respectively; n = 6), which demonstrated the functionality of isolated BBMVs. No changes were observed in the Na^+-dependent D-glucose uptake between WKY and L-carnitine-treated WKY rats at the equilibrium time (30 min; 80 ± 0.6 vs. 78 ± 0.5 pmol/mg protein, respectively; n = 6). Moreover, the treatment with L-carnitine did not alter the intravesicular volume, as estimated from D-glucose distribution at equilibrium (0.8 ± 0.06 vs. 0.78 ± 0.05 μL/mg protein for control and treated rats, respectively).

L-Carnitine Uptake by BBMVs Prepared from WKY and L-Carnitine-Treated WKY Rats

Figure 1 shows the time course of L-carnitine uptake into BBMVs prepared from kidney cortex of WKY and L-carnitine-treated WKY rats. The presence of 100 mmol/L NaCl induced a linear uptake of L-carnitine for 20 s and an overshoot at 5 min in both groups of animals, indicating the presence of an active, Na^+-dependent uptake. In contrast, the uptake in the presence of choline chloride was slow and showed no overshoot in any of the rat groups. Long-term L-carnitine treatment produced a significant decrease in the transport in the presence of sodium at 10, 20, and 30 s, and 1 and 5 min. However, the transport in the absence of a Na^+-gradient was similar in both animal groups. The initial rate of Na^+-dependent transport was significantly lower in treated than in control rats (0.8 ± 0.05 vs. 1.4 ± 0.06 pmol/s per mg protein, respectively, $p < 0.01$); similarly, the magnitude of the accumulation ratio (i.e., the ratio of the transport at overshoot, 5 min, to that at the equilibrium time, 180 min) also diminished in L-carnitine-treated rats (1 ± 0.1-fold vs. 1.95 ± 0.2-fold for treated and untreated WKY rats, respectively, $p < 0.01$). On the other hand, the uptake of L-carnitine at equilibrium was identical in the presence and absence of a Na^+ gradient and was not altered by the treatment.

The effect of membrane electrical potential on L-carnitine uptake is shown in Table III. As expected, an outside-directed K^+ gradient produced an increase in the Na^+-dependent L-carnitine transport in both groups of animals at 20 and 60 s. However, the differences in L-carnitine transport observed between both experimental groups were not abolished in these conditions.

Figure 2 shows the relationship between the external L-carnitine concentration (ranging from 1 to 100 μmol/L) and the measured uptake in WKY and L-carnitine-treated WKY

Table II. Enzyme Activities in Homogenate and Brush-Border Membrane Vesicles (BBMVs) Prepared from Control and L-Carnitine-Treated (LC) Wistar–Kyoto Rats

Enzyme	Homogenate sp ac[a]		BBMVs sp ac		Enrichment[e]		Recovery[f]	
	Control	LC	Control	LC	Control	LC	Control	LC
Alkaline phosphatase[b]	0.25 ± 0.05	0.22 ± 0.02	3.3 ± 0.1	2.9 ± 0.1	12 ± 0.6	13 ± 0.7	34 ± 3	43 ± 3
Na^+–K^+–ATPase[c]	12.1 ± 0.4	11.2 ± 0.2	7.2 ± 0.1	7.1 ± 0.3	0.60 ± 0.01	0.73 ± 0.09	2.0 ± 0.2	1.9 ± 0.1
Acid phosphatase[c]	47 ± 3	46 ± 1	115 ± 2	110 ± 10	2 ± 0.1	2.1 ± 0.1	4 ± 1	4 ± 1
Succinate dehydrogenase[d]	25 ± 7	27 ± 11	4 ± 1	4 ± 1	0.3 ± 0.01	0.32 ± 0.01	0.2 ± 0.02	0.19 ± 0.02

[a] sp ac = Specific activity of enzymes. Expressed in [b]μmol para-nitrophenol formed/min per mg protein; [c]nmol para-nitrophenol formed/min per mg protein; [d]μmol fumarate formed/min per mg protein.
[e] Enrichment = Ratio of the specific activity of brush-border membranes to that of the homogenate.
[f] Recovery = Total activity in brush-border membranes as a percentage of the total activity in the homogenate.
Values are presented as means ± SE for nine different preparations.

rats, in the presence of 100 mmol/L NaCl. Kinetic parameters derived from total L-carnitine fluxes are shown in Table IV. L-carnitine treatment produced a significant reduction in the V_{max} in treated rats (by 52%) when compared to untreated ones. However, no differences were noted in the K_m and K_d values between both groups of animals.

To define the specificity of the carrier-mediated L-carnitine transport, the effect of adding various carnitine analogues (acetyl-L-carnitine, propionyl-L-carnitine and palmitoyl-L-carnitine) in the incubation medium was examined (Fig. 3). Results show that L-carnitine uptake was inhibited in the presence of all these carnitine analogues in both experimental groups, whereas no inhibition was obtained in the presence of D-glucose.

Effect of Medium Osmolarity on L-Carnitine Uptake

To determine whether L-carnitine uptake occurs inside an osmotically sensitive intravesicular space, BBMVs were prepared from WKY and L-carnitine-fed rats, and the intravesicular space was reduced by increasing the medium osmolarity with mannitol. As shown in Fig. 4, L-carnitine uptake at equilibrium (180 min) was directly proportional to the reciprocal of medium osmolarity in BBMVs from control, WKY rats. At infinite osmolarity, there is a minimal binding, which indicates that L-carnitine transport was due to the transport into the intravesicular space. Although not shown, a similar relationship was found in treated rats.

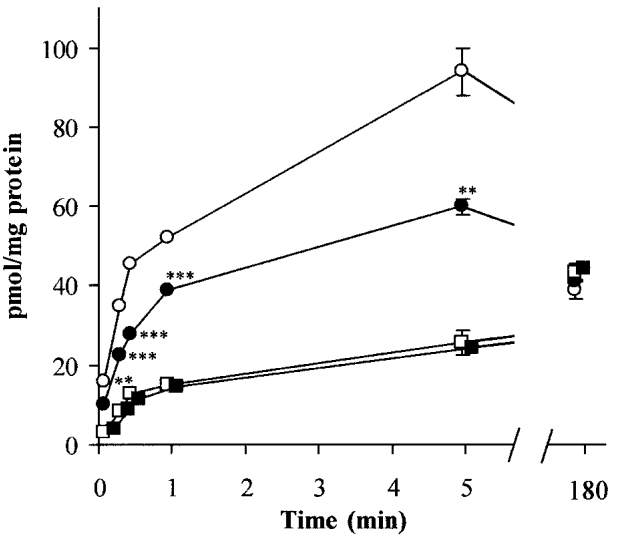

Fig. 1. Time course of L-carnitine uptake in renal brush-border membrane vesicles prepared from control (open symbols) and L-carnitine-treated (closed symbols) Wistar–Kyoto rats in the presence of 100 mmol/L of either NaCl (circles) or choline chloride (squares). Values represent means ± SEM of nine different preparations. **p < 0.01; ***p < 0.001. When not given, SEM bars were smaller than the symbol used.

Determination of the Relative Abundance of OCTN2 in BBMVs

Figure 5A shows Western blot analysis of OCTN2 in BBMVs obtained from renal cortex of control and L-carnitine-treated WKY rats. The antibody recognized a single band of about 70 kDa in both groups. The densitometric analysis of four separate assays (Fig. 5B) indicated a significant reduction in the abundance of OCTN2 in membranes isolated from L-carnitine-fed rats when compared to untreated animals. Figure 5C shows the hybridization signal obtained when the membrane used in Fig. 5A was washed and incubated with anti-actin antibody. This antibody recognized a single band of 45 kDa without significant abundance differences between BBMVs isolated from both groups of animals.

Table III. Effect of Membrane Electrical Potential on Na^+-Dependent L-Carnitine Uptake in Brush-Border Membrane Vesicles (BBMVs) Prepared from Control and L-Carnitine-Treated (LC) Wistar–Kyoto Rats

Condition	L-carnitine uptake (pmol/mg protein)		
	20 s	60 s	180 min
K^+-preloaded BBMVs[a]			
Control	43 ± 3	73 ± 8	48 ± 6
LC	32 ± 2**	55 ± 6**	46 ± 5
Mannitol-preloaded BBMVs[b]			
Control	36 ± 1	52 ± 2	40 ± 3
LC	23 ± 1**	39 ± 2**	40 ± 3

Note: Membrane vesicles were prepared in (mmol/L) [a]100 KCl, 100 mannitol, 0.1 $MgSO_4$, and 20 HEPES/Tris, pH 7.4 (inside negative diffusion potential) or [b]300 mannitol, 0.1 $MgSO_4$, and 20 HEPES/Tris, pH 7.4. Values are means ± SEM for three different preparations.
** p < 0.01 compared with the respective control group.

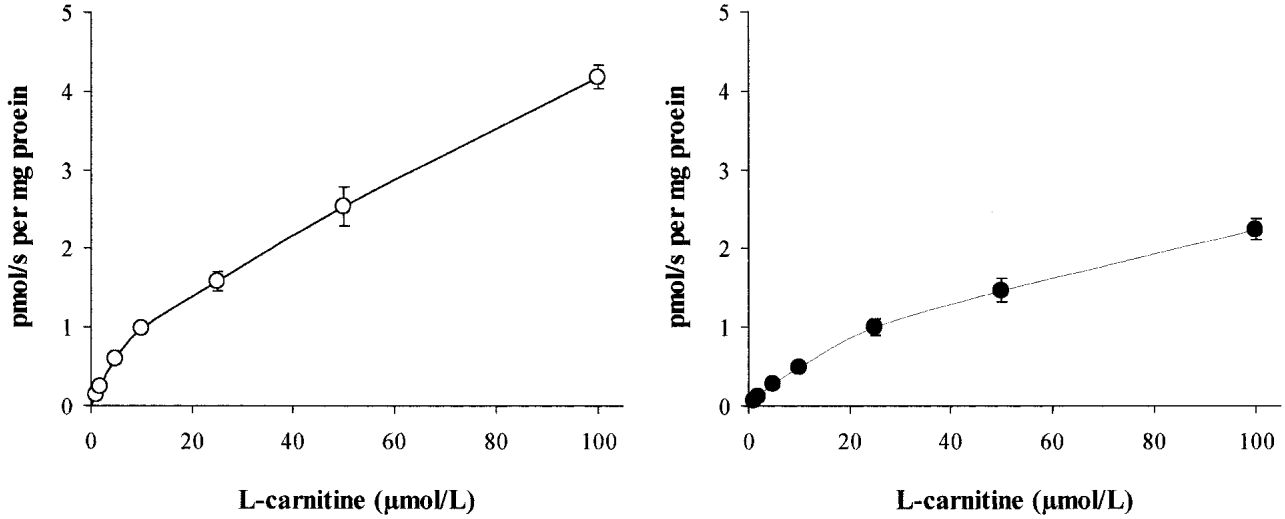

Fig. 2. Kinetics of L-carnitine uptake in renal brush-border membrane vesicles prepared from control (open symbols) and L-carnitine-treated (closed symbols) Wistar–Kyoto rats. Total fluxes were measured at 20 s of incubation at the L-carnitine concentrations given on the abscissa. Kinetic analysis was performed as described in the Materials and Methods. Values represent means ± SEM of six independent experiments. When not given, SEM bars were smaller than the symbol used.

Table IV. Kinetic Parameters of L-Carnitine Uptake in Brush-Border Membrane Vesicles Prepared from Control and L-Carnitine-Treated (LC) Wistar–Kyoto Rats

Parameter	Control	LC
V_{max} [a]	2.5 ± 0.09	1.2 ± 0.12***
K_m [b]	21 ± 2.6	24 ± 2.2
K_d [c]	0.014 ± 0.005	0.012 ± 0.001

Values ([a]pmol/s per mg protein; [b]μM; [c]nL/s per mg protein) are means ± SE for six different preparations.
*** $p < 0.001$ compared with the control group.

DISCUSSION

In our study, untreated rats excreted less than 1% of the filtered free carnitine, a value within the range of other reported values (16,25–27). This indicates that under physiologic conditions, the kidney reabsorbs about 99% of filtered carnitine in a strongly regulated process, as the amount of serum carnitine is maintained without appreciable loss in the urine. On the contrary, the treatment of WKY rats with L-carnitine for 8 weeks induced an increase in the urinary excretion of carnitine (mainly free carnitine), as the result of a reduced renal tubular free carnitine reabsorption rate. These results are in agreement with previous work reporting increases of about 3- to 4-fold in the levels of serum carnitine

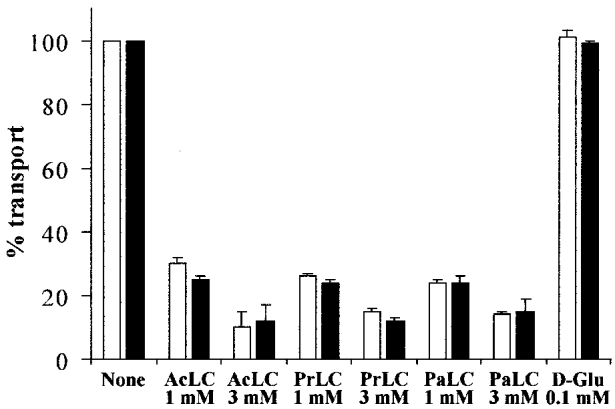

Fig. 3. Effect of different substrates on the uptake of 50 μmol/L L-carnitine in renal brush-border membrane vesicles prepared from control (open bars) and L-carnitine-treated (closed bars) Wistar–Kyoto rats. Uptake was measured at 30 s of incubation in the presence of 1 and 3 mmol/L of acetyl-L-carnitine (AcLC), propionyl-L-carnitine (PrLC), palmitoyl-L-carnitine (PaLC), and 0.1 mmol/L D-glucose. Values represent means ± SEM of five different preparations and are expressed as a percentage of the values obtained in the absence of inhibitors.

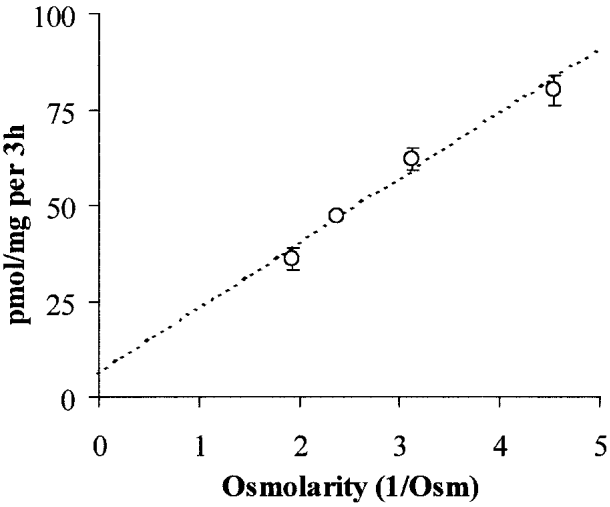

Fig. 4. The effect of incubation medium osmolarity on Na^+-dependent L-carnitine uptake in renal brush-border membrane vesicles prepared from control Wistar–Kyoto rats. Values represent means ± SEM of four different preparations. Regression line ($y = 6.1 + 17x$, $r = 0.971$) was calculated by the least-squares method. A similar relationship was noted for L-carnitine-treated Wistar–Kyoto rats.

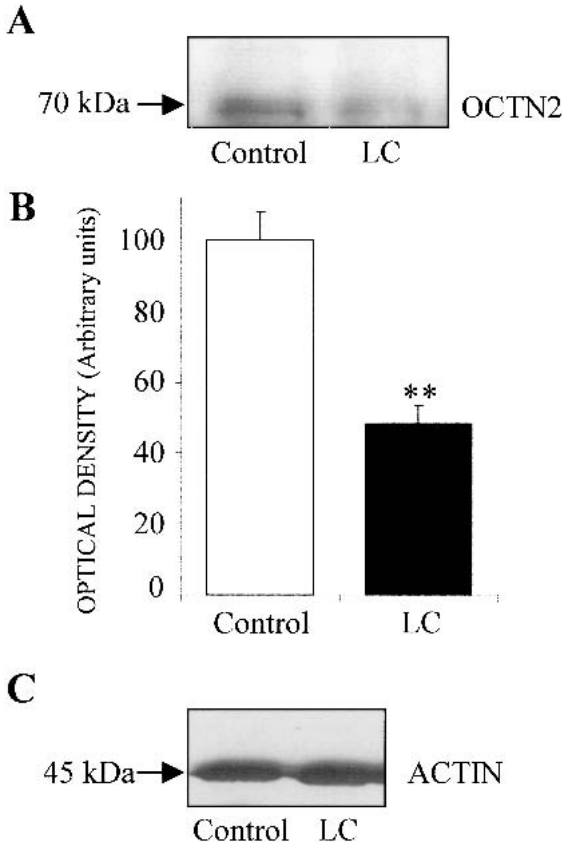

Fig. 5. A, Western blot analysis of OCTN2 in brush-border membrane vesicles prepared from renal cortex of control and L-carnitine-treated (LC) Wistar–Kyoto rats. The antibody recognized an immunoreactive protein of about 70 kDa. B, Relative abundance measured by optical densitometry. Values represent means ± SE of four separate experiments (**$p < 0.01$). C, Immunoblot obtained when the membrane used in (A) was washed and incubated with anti-actin antibody. This antibody recognized a single band of 45 kDa without significant abundance differences between control and L-carnitine-fed Wistar–Kyoto rats.

after the oral administration of this compound (16,26). In addition, a high increase in the urinary excretion of carnitine has also been found in rats after intravenous (28) or intraperitoneal (29) administration of L-carnitine, demonstrating a direct relationship between the administration in large doses and its urinary excretion.

Although it is not clear which nephron segments are accountable for the lower carnitine renal reabsorption rate in treated rats, we report in this study a decrease in the proximal tubule carnitine reabsorption. Initial rates and maximal overshoot levels of Na^+-dependent L-carnitine transport were significantly reduced (by 43% and 49%, respectively) in BBMVs prepared from L-carnitine-treated rats compared with the corresponding values in control WKY rats. This reduction was not due to variations in vesicle preparations, since the purification and the size of BBMVs were similar in both experimental groups. When L-carnitine renal transport was measured in the absence of a sodium gradient, no differences were observed between both animal groups (Fig. 1). In addition, carnitine treatment had no effect on Na^+-dependent D-glucose renal transport, which indicates a specific effect of the treatment on the Na^+-carnitine cotransporter.

These results are in agreement with those obtained by Rebouche and Mack (26), who observed a decrease in Na^+-dependent L-carnitine transport in rat kidney with high levels of dietary carnitine (1% of diet for 10 days). Nevertheless, they also found a reduction in the Na^+-independent L-carnitine transport.

Available evidence indicates that OCTN2 is responsible for the Na^+-dependent, high affinity carnitine transport in the plasma membrane of various tissues including the kidney (4,8,9). Therefore, we evaluated the kinetic properties and substrate specificity of OCTN2, in order to know the mechanism(s) involved in the decrease in the Na^+-dependent L-carnitine renal transport after the treatment with carnitine. For the kinetic analysis, we measured initial influx at concentrations ranging from 1 to 100 μmol/L. Results revealed a reduction of 52% in the maximal transport capacity (V_{max}) in BBMVs prepared from L-carnitine-treated rats compared to WKY rats. No changes were found in the affinity of OCTN2, since both groups showed similar K_m values. In addition, the value of K_d, reflecting the linear component of the transport, was not affected by L-carnitine administration (Table IV).

The V_{max} value in control rats (2.5 pmol/s per mg protein) is similar to that reported by Stieger *et al.* (6) in rat kidney BBMVs but lower than that by Rebouche and Mack (26). Moreover, K_m values (about 20 μM) are in accordance with previous values obtained for the transport of L-carnitine through OCTN2 in rat kidney BBMVs (6), human kidney cell line (7), isolated rat skeletal muscle plasma membrane vesicles (30) and mouse renal BBMVs (8). Lower values have been found in kidney HEK293 cells expressing human OCTN2 (31–33), human fibroblasts (32), Chinese hamster ovary cells transfected with OCTN2 cDNA (34), oocytes expressing human OCTN2 (35) and in plasma membrane vesicles from HEK cells transfected with human OCTN2 (9).

The effect of various L-carnitine acyl-derivatives on L-carnitine transport was also examined in BBMVs from both groups of animals. As previously shown (6–8,30), we found that the carnitine derivatives, L-acetylcarnitine, L-propionylcarnitine and L-palmitoylcarnitine, *cis*-inhibited carnitine transport, with a similar inhibition pattern in control and treated rats (Fig. 3). However, the presence of D-glucose in the medium had no effect on L-carnitine transport.

Although there are a lot of papers regarding the functional and molecular characterization, and the distribution of OCTN2 (4,9), the regulation of this carnitine transporter remains unknown. The observed decrease in V_{max} values in L-carnitine-treated rats indicates a downregulation in the renal proximal tubular transport of carnitine due to the presence of high levels of the substrate, which contributes to the higher urinary carnitine excretion observed in these animals. This finding might be the result of a reduction in the number of OCTN2 molecules and/or to a decrease in the turnover of the transporter. To examine this possibility, we performed Western blot analysis in renal brush-border membranes. A single band of about 70 kDa was detected in both groups of animals, in agreement with the molecular weight reported previously in BBMVs from mouse kidney (8), as well as in homogenates prepared from mouse kidney, heart and skeletal muscle (5,8). Results of blot densitometry demonstrated that the density of OCTN2 protein is approximately 52% lower in BBMVs from L-carnitine-fed rats, a similar reduction to that of V_{max}. Therefore, these results suggest that the lower renal

L-carnitine transport observed in BBMVs from treated rats is directly related to a lower number of OCTN2 molecules, thus existing a down-regulation of the transporter induced by the treatment.

In addition, it has been proved that carnitine transport in kidney (6,8) and skeletal muscle (30) depends on the sodium electrochemical potential. Therefore, and since the transmembrane electrical potential could be altered in apical membranes from L-carnitine-treated rats, we studied the influence of membrane potential on the activity of OCTN2. In the presence of an inside negative diffusion potential (i.e., vesicles preloaded with K^+), the Na^+-dependent L-carnitine transport was higher in both groups of animals, indicating that OCTN2 activity is potential-sensitive. Moreover, the observed changes in L-carnitine transport in treated rats remained in K^+-preloaded BBMVs, which indicates that alterations in the transmembrane electrical potential are not responsible for the impaired L-carnitine absorption observed in these rats.

In conclusion, we have found a decrease in the transport of L-carnitine in renal BBMVs prepared from L-carnitine-treated compared with untreated WKY rats, which is correlated with a reduction in the density of OCTN2 molecules. These results suggest a translational control of OCTN2 expression in the kidney when high levels of its substrate are present, although changes in protein expression at a transcriptional level cannot be excluded.

The molecular mechanisms underlying the downregulation of L-carnitine transport in the renal proximal tubule after the treatment with carnitine must be investigated in more detail. Under normal conditions, carnitine transport from blood to the renal tubular cells is not saturated. However, the long-term administration of L-carnitine produces an increase in the serum carnitine concentration and the renal transport of L-carnitine is saturated. The physiologic relevance of these findings is unclear but it is very important, considering the interest in the therapeutical use of L-carnitine in the cardiovascular disease. It is possible that the downregulation of L-carnitine transport across the renal membrane prevents excessive or even toxic accumulation of L-carnitine in the renal tubular cell in the presence of high levels of carnitine in the blood.

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