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Functional activity of L-carnitine transporters in human airway epithelial cells

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ABSTRACT

Carnitine plays a physiologically important role in the β-oxidation of fatty acids, facilitating the transport of long-chain fatty acids across the inner mitochondrial membrane. Distribution of carnitine within the body tissues is mainly performed by novel organic cation transporter (OCTN) family, including the isoforms OCTN1 (SLC22A4) and OCTN2 (SLC22A5) expressed in human. We performed here a characterization of carnitine transport in human airway epithelial cells A549, Calu-3, NCl-H441, and BEAS-2B, by means of an integrated approach combining data of mRNA/protein expression with the kinetic and inhibition analyses of L-[^{3}H]carnitine transport. Carnitine uptake was strictly Na^+-dependent in all cell models. In A549 and BEAS-2B cells, carnitine uptake was mediated by one high-affinity component ($K_m < 2$ μM) identifiable with OCTN2. In both these cell models, indeed, carnitine uptake was maximally inhibited by betaine and strongly reduced by SLC22A5/OCTN2 silencing. Conversely, Calu-3 and NCl-H441 exhibited both a high ($K_m \sim 20$ μM) and a low affinity ($K_m > 1$ mM) transport component. While the high affinity component is identifiable with OCTN2, the low affinity uptake is mediated by $ATB^{0,+}$, a Na^+, and Cl^--coupled transport system for neutral and cationic amino acids, as demonstrated by the inhibition by leucine and arginine, as well as by SLC6A14/$ATB^{0,+}$ silencing. The presence of this transporter leads to a massive accumulation of carnitine inside the cells and may be of peculiar relevance in pathologic conditions of carnitine deficiency, such as those associated to OCTN2 defects.

1. Introduction

Carnitine (β-hydroxy-γ-trimethylaminobutyrate) is a small, highly polar zwitterionic compound involved in many physiological processes. Among the others, it is known to facilitate the transport of long-chain fatty acids across the inner mitochondrial membrane to allow their β-oxidation, the main source of energy for tissues such as skeletal and cardiac muscle. Inherited defects of all the steps involved in carnitine cycle are transmitted as autosomal recessive traits in humans, confirming the biological relevance of this molecule at both patho- and physiological level [1]. Carnitine cannot be strictly considered an essential nutrient in healthy adults, since it can be both synthesized endogenously by human liver, kidney, and brain [2] and absorbed in the intestinal tract from dietary sources [3].

Appropriate systemic and tissue concentrations of carnitine are mainly maintained by membrane transporters that regulate intestinal absorption, tissue distribution, and renal reabsorption/excretion [4]. Three plasma membrane transporters of carnitine have been identified in human to date, i.e. novel organic cation transporters (OCTNs) OCTN1 (SLC22A4), OCTN2 (SLC22A5), and CT2 (SLC22A16) [5]. In normal tissues, however, only OCTN1 and OCTN2 are ubiquitously expressed, while CT2 is primarily expressed in the testis, kidney, and hematopoietic cells [6].

OCTN1, which is strongly expressed in renal epithelium and, at a lower level, in a wide variety of tissues and cell lines of human origin [7], mediates a bidirectional organic cation transport in a pH-dependent manner [8]. In intact cell systems, it was initially proposed that the model substrate for this transporter is tetraethylamonium (TEA) [9]. More recently, it has been found that, under physiological conditions, OCTN1 is involved in the intracellular accumulation of the mushroom metabolite ergothioneine (ET), an antioxidant which is now considered its specific substrate [10,11]; it is also involved in the transport of acetylcholine [12] and in the excretion of cationic xenobiotics from renal epithelium [7,8].

Despite that OCTN1 could transport carnitine [8,13,14], the main role in the maintenance of carnitine homeostasis is played by OCTN2 which is involved in intestinal absorption, distribution to tissues, and

Abbreviations: $ATB^{0,+}$, amino acid transporter $B^{0,+}$; EBSS, Earle's Balanced Salt Solution; OCTN, novel organic cation transporter; PBS, phosphate-buffered saline solution.

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renal excretion/reabsorption. OCTN2 expression is not limited to polarized cells of intestine, kidney, placenta, and mammary gland but has been found in many other tissues such as liver, heart, testis, skeletal muscle, lung and brain, guaranteeing carnitine absorption and distribution within the entire organism [15]. OCTN2 catalyzes a Na^+-dependent high-affinity transport with apparent K_m ranging between 8 and 80 μM, depending on the tissue [12].

Functional defect of OCTN2 due to genetic mutations causes systemic primary carnitine deficiency (CDSP, MIM 212140), an autosomal recessive disorder characterized by urinary carnitine wasting, that results in low serum carnitine levels and decreased intracellular carnitine accumulation [1,16], with consequent impairment of fatty acid oxidation. The clinical presentation of the disease may include failure to thrive, respiratory insufficiency, vomiting, progressive cardiomyopathy, skeletal myopathy, hypoglycemia, and hyperammonemia [17]. Since CDSP patients respond to carnitine supplementation, it has been postulated that intracellular carnitine supply in these cases can be performed by transporters other than OCTN2; among them, the amino acid transporter $B^{0,+}$ ($ATB^{0,+}$) has been described to perform a low-affinity transport of L-carnitine (Km ~ 800 μM). This transporter exhibits much higher concentrative capacity than OCTN2 because of its energization by transmembrane gradients of Na^+ and Cl^-, as well as by membrane potential [18]. $ATB^{0,+}$ is principally expressed in intestine, lung, and mammary gland and belongs to the gene family of Na^+- and Cl^--coupled transporters for a variety of compounds, such as amino acids, neurotransmitters, and osmolytes.

In airways, OCTNs are highly expressed at the apical side of airway epithelial cells in tracheal tissue, as well as in alveolar epithelial cells [7,19,20]. In these latter cells, carnitine is involved in the production of pulmonary surfactant [21], the mixture of phospholipids, cholesterol, and proteins that reduces the surface tension at the air–liquid interface of the lung, preventing alveolar collapse and allowing for normal gas exchange. At early stages of life, carnitine biosynthesis is less efficient than in adults and preterm neonates do not synthesize sufficient amounts of carnitine with respect to term infants [22]. Antenatal carnitine administration has been, hence, proven effective in inducing pulmonary surfactant production and lung maturation in both fetal rats and humans [21,23]; in addition, Ozturk et al. reported that exogenous carnitine supply to mothers who have the risk of premature delivery and to the preterm newborns might prevent or decrease the severity of respiratory distress syndrome (RDS) [24], the most severe complication observed in preterm infants and the most frequent cause of mortality of immature infants. L-carnitine supplementation can also be important for other lung diseases. In chronic obstructive pulmonary diseases (COPD) patients, L-carnitine administration can improve exercise tolerance and inspiratory muscle strength [25]. It has been also noted that L-carnitine reduces leukotriene synthesis through inhibition of lipoxygenase enzyme [26] and improves the pulmonary function test of children with moderately persistent asthma [27].

Thus far, little is known about the functional operation of carnitine transporters in the airways. The purpose of the present study is, therefore, to characterize carnitine transport mechanisms in cell models of human airway epithelium, i.e. tracheobronchial Calu-3 and BEAS-2B, bronchiolar-alveolar NCI-H441 and alveolar type II A549 cells.

2. Materials and methods

2.1. Cell cultures

A549, Calu-3, NCl-H441, and BEAS-2B human cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Alveolar carcinoma A549 and normal bronchial epithelial BEAS-2B cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose) supplemented with sodium pyruvate (1 mM). NCl-H441, obtained from lung papillary adenocarcinoma, were cultured in RPMI-1640 (ATCC) and used at passages 36–41. Calu-3 cells, obtained from a human lung adenocarcinoma and derived from serous cells of proximal bronchial airways, were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with sodium pyruvate (1 mM) and used at passages 45–53. For all cell types, medium was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were routinely cultured under physiological conditions (37.5 °C, 5% CO_2, 95% humidity) in 10-cm diameter dishes.

Chinese hamster ovary (CHO) cells transfected with OCTN2 (CHO-OCTN2), kindly provided by Dott. Longo N.,were grown in Ham F12 medium supplemented with 6% fetal bovine serum as previously described [28].

2.2. Uptake studies

For uptake assay, 3×10^4 cells were seeded onto 96-well trays (Falcon) and uptake was measured when the confluence was reached. After two washes in pre-warmed transport buffer (Earle's Balanced Salt Solution (EBSS) containing (in mM) 117 NaCl, 1.8 $CaCl_2$, 5.3 KCl, 0.9 NaH_2PO_4, 0.8 $MgSO_4$, 5.5 glucose, 26 Tris/HCl, adjusted to pH 7.4), cells were incubated in fresh transport buffer containing L-[^{3}H]carnitine (2 μCi/ml) for the times detailed in each experiment (see figure legends). For a sodium-free EBSS, 117 mM NaCl was replaced with equimolar N-methyl-D-glucamine chloride, while in Cl-free EBSS sodium and potassium chloride were substituted with the corresponding gluconate salts. Where indicated, the inhibitors were added to the transport buffer at the indicated concentrations. At the indicated times, transport buffer containing the radiolabeled substrate was removed, and the experiment terminated by two rapid washes (<10 s) in ice-cold 300 mM urea. Cell monolayers were extracted in ethanol and the radioactivity in cell extracts determined with Wallac Microbeta Trilux^2 liquid scintillation spectrometer (Perkin Elmer, Monza, Italy). Protein content was determined directly in the well using a modified Lowry procedure [29]. L-carnitine uptake is expressed as nmol or pmol/mg of protein, as indicated. For the determination of L-carnitine intracellular concentration (see Fig. 9), cell volume was estimated from the distribution space of [^{14}C]urea, as already described [30]. Briefly, [^{14}C]urea (0.5 mM; 2 μCi/ml) was added to A549 and Calu-3 cells during the last 10 min of incubation in transport buffer. The incubations were terminated by two rapid washes (<10 s) in ice-cold 300 mM urea and radioactivity in cell extracts was determined as described above. Calculated cell volumes corresponded to 9.3 ± 0.14 μl/mg of protein and 8.4 ± 0.9 μl/mg of protein for A549 and Calu-3, respectively (not shown).

The apparent kinetic parameters K_m (Michaelis constant) and V_{max} (maximum transport rate) of L-carnitine uptake were calculated by non-linear regression fitting, according to the following Michaelis–Menten equations:

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \quad (1)$$

for a single saturable component plus diffusion, where v is the initial influx;

$$v = \frac{V_{max1} \times [S]}{K_{m1} + [S]} + \frac{V_{max2} \times [S]}{K_{m2} + [S]} \quad (2)$$

for two saturable transport components, where indices 1 and 2 indicate the high- and low-affinity components, respectively.

The following equation was employed to describe a competitive inhibition of L-carnitine transport:

$$v = v_0 - \frac{I_{max} \times [I]}{[I]_{0.5} + [I]} \quad (3)$$

where v is the initial influx, v_0 is the uptake in the absence of the inhibitor, I_{max} is the maximal inhibition, and $[I]_{0.5}$ is the inhibitor concentration required for half-maximal inhibition.

2.3. qRT-polymerase chain reaction

For the analysis of mRNA expression 1 μg of total RNA, extracted with GenElute Mammalian Total RNA Miniprep Kit (Sigma–Aldrich, Milano, Italy), was reverse-transcribed and 40 ng of cDNA were amplified as described previously [31], employing the following forward and reverse primers for SLC6A14/ATB$^{0,+}$, 5′ GCT GCT TGG TTT TGT TTC TTC TTG GTC 3′ and 5′ GCA ATT AAA ATG CCC CAT CCA GCA C 3′. The expression of SLC22A4/OCTN1, SLC22A5/OCTN2 and the housekeeping gene RPL15 (ribosomal protein like 15) were monitored employing specific TaqMan® Gene Expression Assays (Life Technologies Italia, Milano, Italy; Cat no. Hs00268200_m1, Hs00929869_m1, and Hs03855120_g1, respectively), according to the manufacturer's instructions. The expression of the gene of interest under each experimental condition was normalized to that of the housekeeping gene, as indicated.

2.4. Western blot analysis

Cells were washed with ice-cold PBS and suspended in Laemmli buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 20% glycerol, 0.2 M DTT), then Western blot analysis was performed as previously described [32] using anti-OCTN1 (1:400; Sigma–Aldrich), anti-OCTN2 (1:1000; Sigma–Aldrich), or anti-ATB$^{0,+}$ (1:1000; Abcam) antibodies in blocking solution. Actin, detected with a polyclonal antibody (1:5000; Sigma–Aldrich) was employed as internal standard. Immunoreactivity was visualized with enhanced chemiluminescence (Millipore, Milano, Italy).

2.5. siRNA transfection

Gene silencing of SLC22A5/OCTN2 and SLC6A14/ATB$^{0,+}$ was performed employing specific FlexiTube short interfering RNA (siRNA) (Cat. no. 1027416) by Qiagen® (Milano, Italy), according to Fast-Forward Transfection protocol provided by the manufacturer. Briefly, cells (1×10^5/ml) were transfected by adding to 1 ml of cell suspension 200 μl of serum-free medium containing HiPerFect Transfection Reagent (9 μl) and 100 nM AllStar Negative Control siRNA (Cat. no. SI03650318; scrambled) or 25 nM each of 4 different SLC22A5/OCTN2 (Cat. no. SI04357920, SI04181499, SI04147752, SI04144861) or SLC6A14/ATB$^{0,+}$ (Cat. no. SI03098529, SI00095739, SI00095732, SI00095725) siRNA. Transfected cells were seeded as required by the experimental plan and maintained under growing conditions for 72 or 96 h, as indicated. Control, untransfected cells were also cultured in parallel.

2.6. Data calculation and statistical analysis

All curve fittings were done using Prism® 5.0 (GraphPad, San Diego, CA), as well as the statistical analyses, performed using Student's t test, one-way ANOVA followed by Bonferroni post hoc test or comparison of fit, as specified. Unless otherwise stated, all experiments were performed in quadruplicate and data presented as mean ± S.E.

2.7. Materials

Fetal bovine serum was purchased from EuroClone (Milano, Italy), while NCI-H441 were from ATCC (Rockville, MD, USA). Carnitine-L-[N-methyl-^{3}H]HCl (80 Ci/mmol) was obtained from Perkin–Elmer (Milano, Italy). The antibody against ATB$^{0,+}$ was from BIOTIME SAS (Siena, Italy). Sigma–Aldrich (Milano, Italy) was the source of the inhibitors, as well as of the anti-OCTN1, anti-OCTN2, and anti-actin antibodies, and unless otherwise specified, of all other chemicals.

3. Results

3.1. Time-dependent accumulation of L-carnitine in A549, BEAS-2B, Calu-3, and NCI-H441 cells

The time course analysis of 1 μM L-[^{3}H]carnitine uptake was performed in the four cell lines. As shown in Fig. 1, substrate uptake increased in a time-dependent manner and was linear at least up to 1 h in all cell models. Moreover, it appeared strictly Na^+-dependent, being completely abolished by the replacement of extracellular sodium with *N*-methyl-D-glucamine (NMDG). The rate of L-carnitine transport was maximal in BEAS-2B cells, with A549 and Calu-3 following; NCI-H441 cells displayed a modest uptake of L-carnitine, with rate values ten times lower than those observed in BEAS-2B cells. Moreover, in A549 and BEAS-2B, an excess of unlabelled carnitine completely suppressed the transport, while a residual activity was still detectable in both Calu-3 and NCI-H441 cells under the same conditions. An incubation time of 30 min was hereafter used for the measurement of L-[^{3}H]carnitine uptake.

3.2. Kinetic analysis of L-carnitine uptake in A549, BEAS-2B, Calu-3, and NCI-H441 cells

We next examined the concentration-dependent uptake of L-carnitine over a wide range of substrate concentrations (from 0.00049 to 1.85 mM). Results of the Na^+-dependent uptake are shown in Fig. 2. In A549 and BEAS-2B cells, the values were best fitted by Eq. (1) (see Materials and methods) and the resulting kinetic parameters

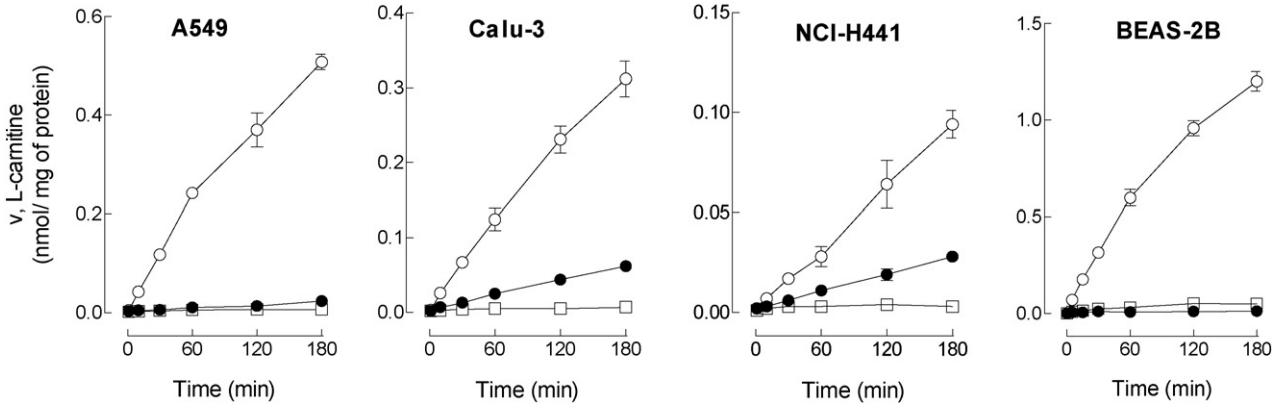

Fig. 1. *Time-dependent accumulation of L-carnitine in A549, Calu-3, NCI-H441, and BEAS-2B cells.* Cells were incubated for the indicated time in transport buffer (see Materials and methods) containing L-[^{3}H]carnitine (1 μM; 2 μCi/ml), in the presence (open circles) or in the absence of Na^+ (open squares). For Na^+-free buffer, NaCl was replaced by an equimolar concentration of N-methyl-D-glucamine chloride. Non-specific binding of the substrate was estimated by measuring the uptake of L-carnitine in the presence of an excess of unlabelled substrate (2 mM) (filled circles). Each point represents the mean ± S.D. of four independent determinations. The experiment has been repeated three times with comparable results.

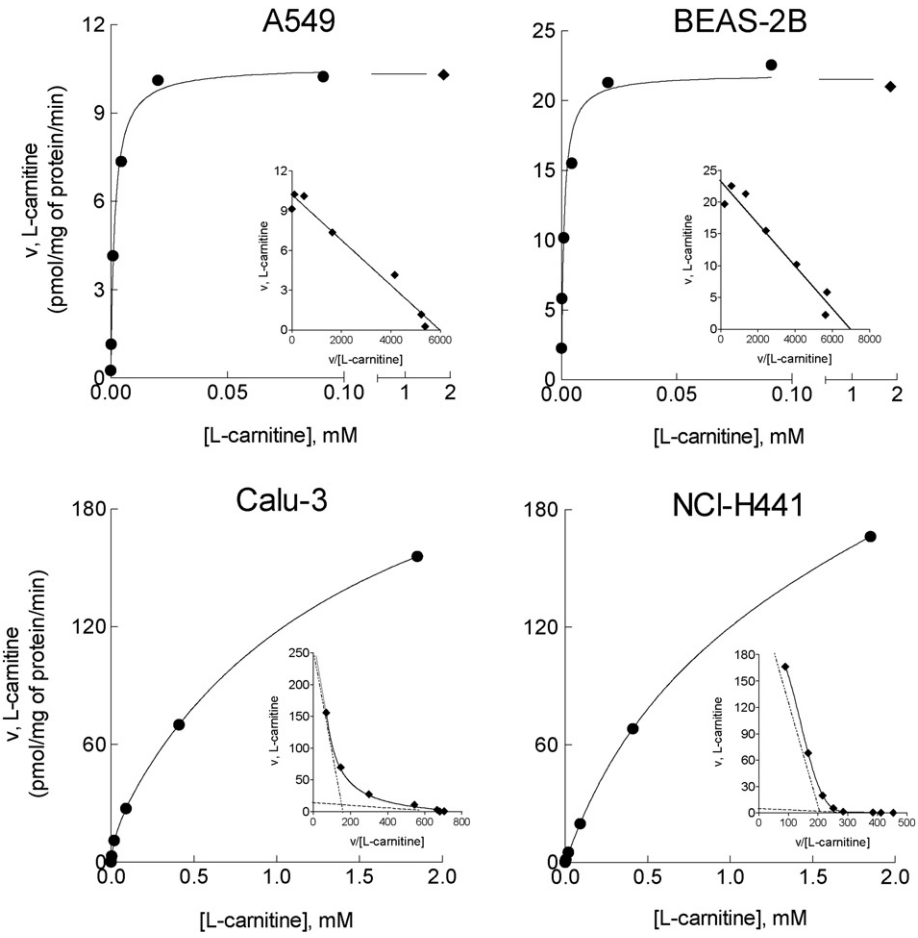

Fig. 2. *Kinetic analysis of L-carnitine uptake in A549, Calu-3, NCI-H441, and BEAS-2B cells.* Cells were incubated in the presence of the indicated concentrations (from 0.49 μM to 1.85 mM) of L-[^{3}H]carnitine (2 μCi/ml) for 30 min, either in the presence or in the absence of Na^+ (not shown). The plot shows the Na^+-dependent component, calculated by subtracting transport data obtained in the absence from those in the presence of Na^+. Nonlinear fitting of the data was performed employing Eq. (1) (A549 and BEAS-2B cells) or Eq. (2) (Calu-3 and NCI-H441 cells) (see Materials and methods). The corresponding kinetic constants for the four cell lines are given in Table 1. Insert in each panel shows the Eadie–Hofstee transformations of the data. For A549 and BEAS-2B, lines represent the linear regression analyses, while, for Calu-3 and NCI-H441, curves represent the nonlinear fitting of the data. Points are mean ± S.D. of three independent determinations. The experiment has been repeated twice with comparable results.

revealed the operation of one high-affinity transport system (Km of 1.7 and 3.3 μM, respectively); consistently, the Eadie–Hofstee plots of the data (inserts) were linear, confirming the operation of a single transporter in both models. On the contrary, in Calu-3 cells, L-carnitine transport data were best fitted by Eq. (2), revealing the presence of two saturable transport components, one displaying a high affinity for the substrate (Km = 25 μM) and the other with a low affinity (Km > 1 mM). Accordingly, the regression analysis of the Eadie–Hofstee plot for carnitine in these cells (insert) appeared nonlinear, confirming the involvement of at least two different transporters. Also, in NCI-H441 cells, transport data were best fitted by Eq. (2) for a high- and a low-affinity component. This latter model presented kinetic features very similar to those of Calu-3. A complete description of the apparent kinetic parameters obtained in all cell models is shown in Table 1.

Table 1
Kinetic parameters describing the uptake of carnitine.
Data have been estimated from the nonlinear regression analysis shown in Fig. 2, employing Eq. (1) for A549 and BEAS-2B and Eq. (2) for Calu-3 and NCI-H441.

	High affinity		Low affinity	
	K_{m1} μM	V_{max1} pmol/mg of protein/min	K_{m2} μM	V_{max2} pmol/mg of protein/min
A549	1.72 ± 0.3	10.23 ± 0.43		
BEAS-2B	3.2 ± 0.4	23.21 ± 0.68		
Calu-3	25.7 ± 1.4	10.7 ± 0.49	1470 ± 250	251.2 ± 15.6
NCI-H441	12.7 ± 4.1	4.53 ± 0.28	1251 ± 10.8	247.7 ± 5.48

3.3. Expression of carnitine transporters in A549, BEAS-2B, Calu-3, and NCI-H441 cells

The expression of SLC22A4/OCTN1, SLC22A5/OCTN2, and SLC6A14/$ATB^{0,+}$ has been next evaluated in A549, NCI-H441, Calu-3, and BEAS-2 cells, in terms of both mRNA (Fig. 3, panel A) and protein (panel B) level. OCTN1 was expressed in all cell models, although less abundant in NCI-H441; $ATB^{0,+}$ was maximally expressed in Calu-3 cells with NCI-H441 following and only barely detectable in A549 and BEAS-2B. The expression of OCTN2 was clearly evident in all cell models.

3.4. Inhibition analysis of L-carnitine uptake in A549, BEAS-2B, Calu-3, and NCI-H441 cells

In order to better address the specific transporters involved in L-carnitine uptake in the different cell models, the uptake of the substrate was measured in the presence of different organic cationic and zwitterionic compounds (Fig. 4). In A549 and BEAS-2B, L-carnitine uptake was maximally inhibited by betaine (substrate of OCTN2) and, to a less extent, by TEA (substrate of OCTN1/2). Ergothioneine (ET), the specific high-affinity OCTN1 substrate [10], was ineffective at 100 μM, while, at higher concentrations (1 mM), caused a significant inhibition of carnitine uptake in both cell models; on the contrary, the cationic amino acid arginine, as well as the neutral amino acids leucine and proline, were completely ineffective. The absence of chloride in the

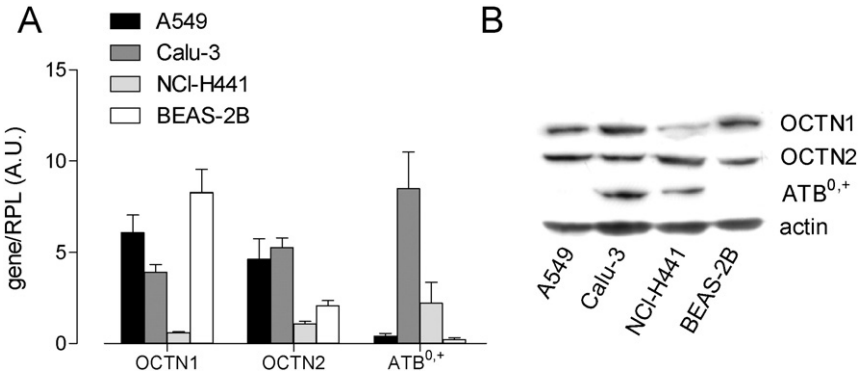

Fig. 3. *Expression of OCTNs and ATB$^{0,+}$ in A549, Calu-3, NCI-H441, and BEAS-2B cells.* Panel A. mRNA levels for OCTNs and ATB$^{0,+}$ were determined through RT-qPCR analysis. After normalization to RPL-15, the expression of SLC22A4/OCTN1, SLC22A5/OCTN2, and SLC6A14/ATB$^{0,+}$ was expressed as the ratio between the expression of the gene of interest and that of the housekeeping gene in each cell model. Data are means ± S.E. of three experiments, each performed in duplicate. Panel B. Protein expression was evaluated in total cell lysates, as described in Materials and methods. A representative Western blot is shown. The experiment was repeated twice with comparable results.

transport buffer did not affect carnitine uptake in these cell models, hence excluding the contribution of ATB$^{0,+}$. In Calu-3 cells, leucine showed the strongest inhibitory effect (more than 60%) among the compounds tested, with arginine following; also, betaine produced a significant inhibition of L-carnitine uptake (about 50%), while TEA and ET (at both concentrations) were ineffective. Carnitine transport was significantly inhibited by the use of Cl–free buffers. The pattern of inhibition in NCl-H441 cells appeared completely comparable to that of Calu-3 cells, since the effects of the different compounds were overlapping. Overall these results point to the operation of OCTN2 in all cell models and to the further contribution of ATB$^{0,+}$ in Calu-3 and NCl-H441 cells.

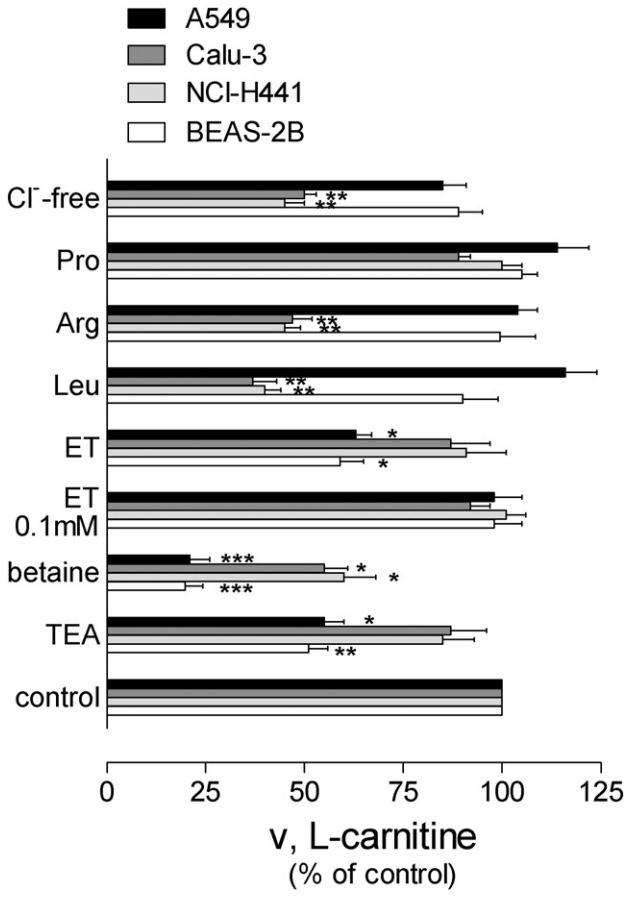

Fig. 4. *Inhibitory effect of organic cationic and zwitterionic compounds on L-carnitine uptake in A549, Calu-3, NCl-H441, and BEAS-2B cells.* The uptake of L-[^{3}H]carnitine (30 min; 1 μM; 2 μCi/ml) was determined in the absence (control) or in the presence of the listed compounds (all tested at the concentration of 1 mM, except for ergothioneine, also used at 0.1 mM). The effect of the Cl-free buffer was evaluated by substituting chloride with gluconate, as described in Materials and methods. The inhibitory effect of each compound is calculated as the residual amount of L-carnitine uptake, expressed as percent of control. Values are mean ± S.E. of three experiments each performed in triplicate. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control with ANOVA.

On the other hand, the inhibitory effect of 1 mM ET in A549 and BEAS-2B cells could suggest the involvement of OCTN1 in carnitine uptake. To verify this hypothesis, an inhibition analysis of L-carnitine uptake by betaine and ET was performed in these cells (Fig. 5, panel A). Results obtained indicated that in A549, both compounds inhibited substrate uptake in a dose-dependent manner, with betaine being more effective ($I_{max} = 99.6 \pm 8\%$) than ET ($I_{max} = 85 \pm 15\%$). The concomitant presence of the two inhibitors, while did not change the maximal inhibition obtained by betaine alone, shifted the inhibition curve towards left. Indeed, the $I_{0.5}$ value calculated upon simultaneous addition of ET and betaine was the half of that obtained with the sole betaine, (from 0.58 ± 0.1 to 0.27 ± 0.04 mM), demonstrating that the two compounds interact with the same transporter, presumably OCTN2. In BEAS-2B, the effect of ET was negligible at low concentrations, while becoming evident at 1 mM; also, in these cells, however, the addition of ET to betaine modified only the value of $I_{0.5}$ (from 0.117 mM in the presence of betaine to 0.063 mM in the presence of betaine + ET), without affecting the I_{max} values (84.2% in the presence of betaine and 82.8% in the presence of betaine + ET). These data suggest that in A549 and BEAS-2B cells, only OCTN2 is operative and point to ergothioneine, when employed at high doses, as a substrate for this transporter. The results shown in Fig. 5 (panel B) by employing CHO cells transfected with OCTN2 (CHO-OCTN2) confirmed this hypothesis, since 1 mM ET significantly inhibited carnitine transport, confirming that, at high doses, ET also interacts with OCTN2 transporter.

In Calu-3 and NCl-H441 cells, the inhibition analysis of 1 μM L-carnitine uptake was carried out employing betaine and leucine as inhibitors. Data shown in Fig. 6 indicate that the inhibitory profile of these compounds is comparable. In Calu-3, the maximal inhibition was 58.4 ± 3.9% and 50.8 ± 2.5% for leucine and betaine, respectively, when added individually; when present in combination, the effects of the inhibitors were additive, with a maximal inhibition up to 85.2 ± 1.9%. Comparable effects were observable in NCl-H441. The inhibitory profile obtained suggests that L-carnitine transport in these cell models is mediated by OCTN2 and by ATB$^{0,+}$, a Na^+- and Cl^--coupled transport system for neutral and cationic amino acids; more precisely, it is likely to assume that OCTN2 accounts for the high affinity component, while ATB$^{0,+}$ represents the low-affinity transporter for L-carnitine uptake.

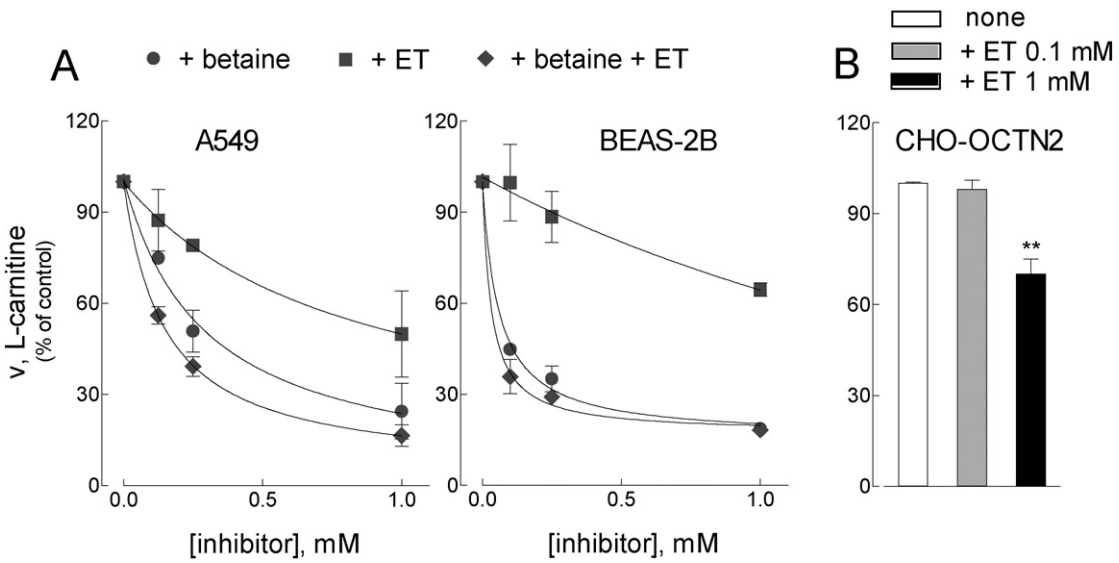

Fig. 5. *Inhibition of L-carnitine uptake by betaine and ergothioneine.* A549 and BEAS-2B (panel A) and transfected CHO-OCTN2 (panel B) cells were incubated for 30 min in the transport buffer containing L-[^{3}H]carnitine (1 μM; 2 μCi/ml) in the absence (none, control) or in the presence of the indicated concentrations of inhibitor. Each point represents the mean ± S.D. of four independent determinations. Data in panel A were fitted by Eq. (3) (see Materials and methods). The experiment has been repeated three times with comparable results. $^{**}p < 0.01$ versus control (none).

3.5. Carnitine transport in silenced cells

In light of these results, we next employed short interfering RNA (siRNA) to define the operation of the different transporters. In A549 (Fig. 7, upper panels), an almost complete suppression of SLC22A5/OCTN2 (80%) was reached after 72 h of incubation (panel A), when a marked reduction of transport activity (>70%) was observed (panel B). Upon SLC22A5/OCTN2 silencing, betaine almost completely lost its inhibitory effect (panel C). Indeed, the inhibition curves obtained in scrambled and SLC22A5 silenced cells were significantly different ($p < 0.01$), with the I_{max} dropping from 9.8 ± 1 to 2.1 ± 0.2 and $I_{0.5}$ from 0.55 ± 0.1 to 0.15 ± 0.06, respectively. These results, beside confirming the prevalent operation of OCTN2 in A549 cells, also validate the efficacy of betaine as inhibitor of OCTN2. A less efficient silencing of SLC22A5/OCTN2 (about 40%) was obtained in BEAS-2B (Fig. 7, panel D), which caused, however, a significant reduction of carnitine transport referable to a decrease of the betaine-sensitive quote (panel E). In both Calu-3 and NCI-H441 cells, also SLC6A14/ATB$^{0,+}$ was silenced beside SLC22A5/OCTN2 (Fig. 8). The expression of both genes was markedly reduced (panels A and C) and, consistently, carnitine transport was lowered (panels B and D). In particular, a 60% and 50% reduction of SLC22A5 and SLC6A14, respectively, caused a decrease of about 40% of carnitine transport in Calu-3 cells. In NCI-H441, a 40% reduction of the expression of both genes caused a comparable decrease of transport activity.

In order to assess if an impairment of the activity of the high-affinity OCTN2 transporter may differently impact on cell models that express only OCTN2 or both OCTN2 and ATB$^{0,+}$, a time course of 30 μM carnitine uptake (roughly corresponding to the plasma concentration) was performed in A549 and Calu-3 cells silenced with SLC22A5/OCTN2

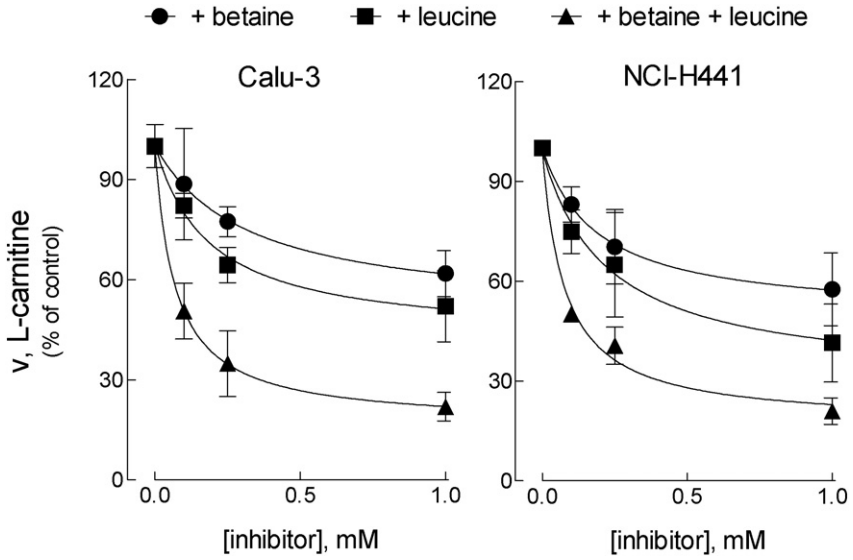

Fig. 6. *Inhibition of L-carnitine uptake by betaine and leucine in Calu-3 and NCI-H441 cells.* Cells were incubated for 30 min in the transport buffer containing L-[^{3}H]carnitine (1 μM; 2 μCi/ml) with the indicated concentrations of inhibitor. Each point represents the mean ± S.D. of four independent determinations. Data were fitted by Eq. (3) (see Materials and methods). The experiment has been repeated three times with comparable results.

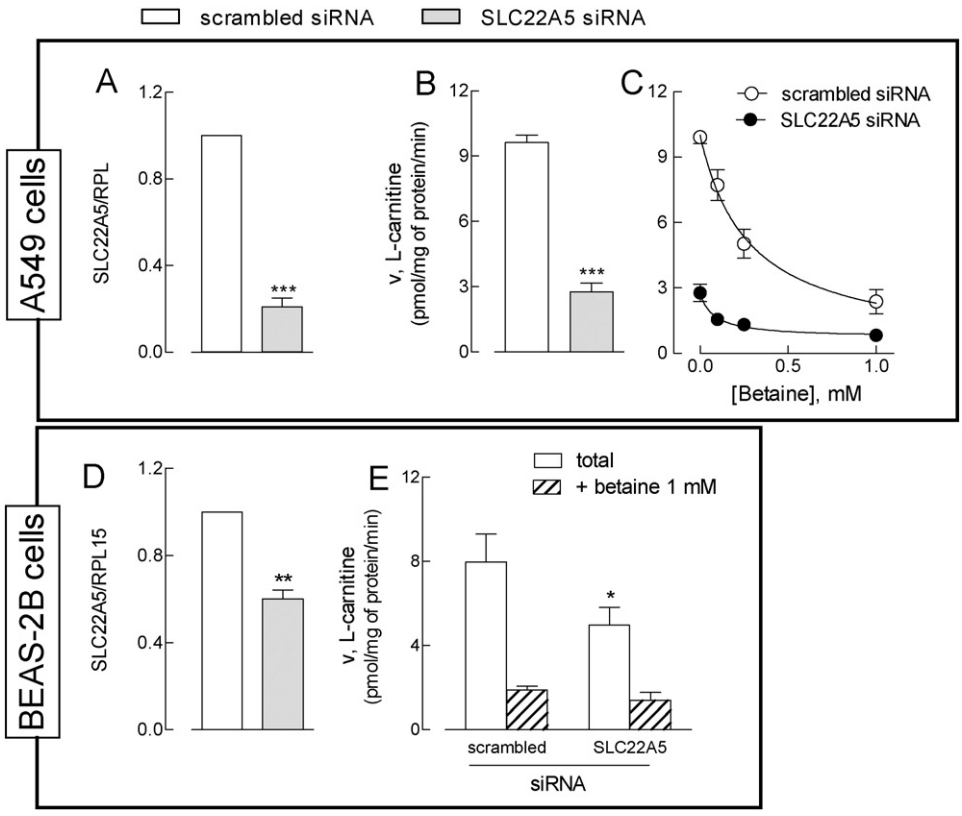

Fig. 7. *SLC22A5/OCTN2 silencing in A549 and BEAS-2B cells.* A549 (panels A, B, and C) and BEAS-2B (panels D and E) were transfected with scrambled or SLC22A5/OCTN2 siRNA for 72 h, as described in Materials and methods. Panels A and D. The expression of SLC22A5/OCTN2 mRNA in silenced cells was assessed by qRT-PCR and shown relatively to that observed in scrambled transfected cells (=1). Data are means ± S.E. of three separate determinations, each performed in duplicate. Panel B. The uptake of L-[^{3}H]carnitine (30 min; 1 μM; 2 μCi/ml) was measured in cells transfected with scrambled or SLC22A5/OCTN2 siRNA. Data are means ± S.E. of three independent experiments, each performed in quadruplicate. Panels C and E. Transfected cells were incubated for 30 min in the transport buffer containing L-[^{3}H]carnitine (1 μM; 2 μCi/ml) with the indicated concentrations of betaine. Each point represents the mean ± S.D. of four independent determinations. The experiments have been repeated twice with comparable results. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs scrambled transfected cells, with Student's t test. Data in Panel C were fitted by Eq. (3) (see section Materials and methods); differences between curves were statistically significant (comparison of fits, see Materials and methods; $^{**}p < 0.01$).

siRNA (Fig. 9). In A549, the intracellular concentration of carnitine was markedly reduced by OCTN2 silencing; on the contrary, in Calu-3 cells, the accumulation of carnitine, much higher than in A549 cells, was not affected by the suppression of OCTN2 transporter. These data suggest that the presence of ATB$^{0,+}$ transporter in Calu-3 cells can compensate for the lack of OCTN2 in supplying cells with extracellular carnitine.

4. Discussion

In the present study, we performed for the first time a complete characterization of carnitine transport in human airway epithelial Calu-3, A549, NCl-H441, and BEAS-2B cells. Our results indicate that OCTN2 is the major contributor to L-carnitine uptake in A549 and BEAS-2B cells, while ATB$^{0,+}$, beside OCTN2, mediates L-carnitine transport in Calu-3 and NCl-H441 cells. Although many *in vitro* studies have assessed the expression of OCTN proteins in lung epithelial cell models, investigations into the functional role of these transporters are currently scarce and, in particular, the kinetic properties of carnitine transport has been not fully explored.

Despite some controversial results [33], many studies have thus far shown that OCTN1 and OCTN2 are highly expressed in the respiratory epithelium of human trachea and bronchi [7,19,20,34]. In particular, both transporters have been localized at the apical membrane of differentiated epithelial cells from trachea and lung parenchima, with OCTN2 showing a relatively stronger expression at the surface of the alveolar type I epithelium [19]. Moreover, the expression of OCTN1/2 proteins has been detected in undifferentiated Calu-3 cells by means of immunocytochemistry and Western blot [35,36], as well as at the apical surface of Calu-3 grown under air–liquid interface conditions [37] and in NCl-H441 cells [38]. In line with these results, we show here that both OCTN1 and OCTN2 mRNA are expressed in all the cell models employed (Calu-3, A549, NCl-H441, and BEAS-2B cells), and consistently, OCTNs proteins are clearly detectable, although OCTN1 is slightly fainter in NCl-H441 (Fig. 3).

Beside the expression of OCTNs, we also provide evidence for the presence of ATB$^{0,+}$ mRNA and protein in Calu-3 cells and, although to a lesser extent, in NCl-H441. This transporter, first described in human epithelial airway cells by Galietta [39], has been reported to be expressed throughout the lung [40]. Conversely, our results clearly indicate that only some airway models (i.e. Calu-3 and NCl-H441) actually express ATB$^{0,+}$, while others, such as A549 and BEAS-2B cells, do not.

In our study, the characterization of the kinetics of L-carnitine transport indicates that A549 and BEAS-2B cells display the most sustained uptake, and that this is mediated by a single high-affinity, sodium-dependent transporter (see Fig. 2). Moreover, in light of the complete inhibition of carnitine uptake by betaine, one of the preferential substrates of OCTN2 [41], we can actually identify the only transporter operative in this model with OCTN2. Conversely, the functional characterization of L-carnitine-mediated transport in Calu-3 and NCl-H441 cells reveals that besides OCTN2, also ATB$^{0,+}$ is operative in these two cell models, in line with expression profile. Indeed, the kinetic analysis indicates the involvement of two saturable components, one with a high affinity for the substrate, identifiable with OCTN2 ($K_m \leq 25$ μM), and the other with a low affinity ($K_m > 1.2$ mM). The marked inhibition

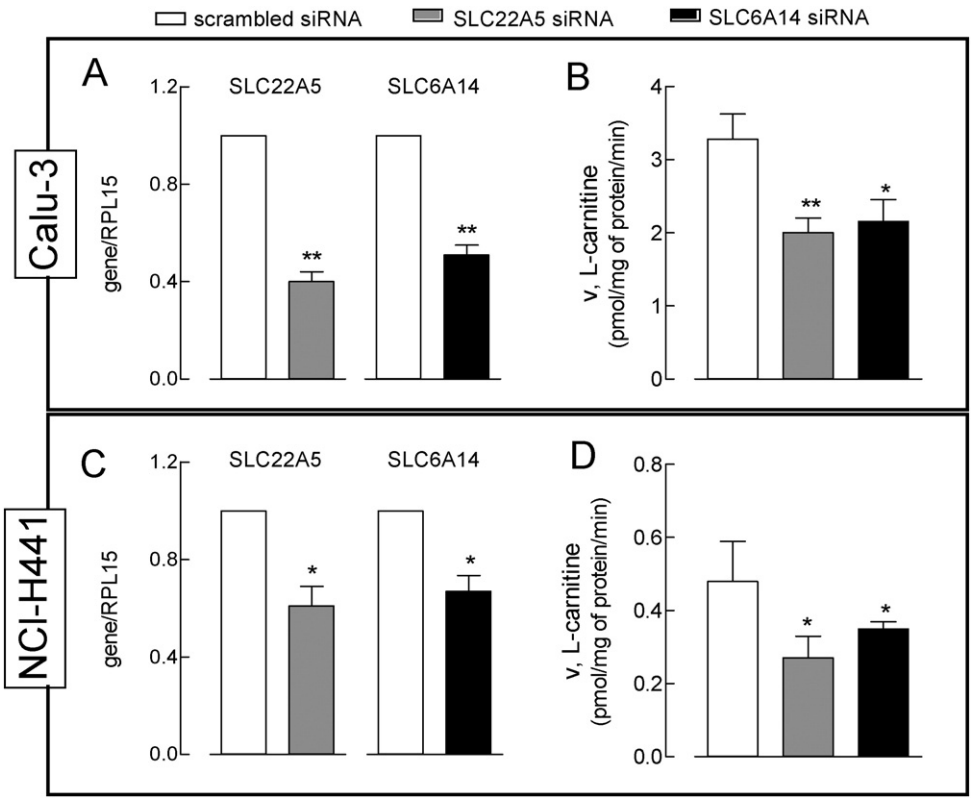

Fig. 8. *SLC22A5/OCTN2 and SLC6A14/ATB$^{0,+}$ silencing in Calu-3 and NCI-H441 cells.* Calu-3 cells (panels A and B) and NCI-H441 (panels C and D) were transfected with scrambled, SLC22A5/OCTN2 or SLC6A14/ATB$^{0,+}$ siRNA for 96 h, as described in Materials and methods. Panels A and C. The expression of SLC22A5/OCTN2 and SLC6A14/ATB$^{0,+}$ mRNA in silenced cells was assessed by qRT-PCR and shown relatively to that observed in scrambled transfected cells (=1). Data are means ± S.E. of three separate determinations, each performed in duplicate. Panels B and D. The uptake of L-[^{3}H]carnitine in silenced cells was measured at 1 μM substrate. Data are means ± S.E. of two independent experiments, each performed in quadruplicate. $^*p < 0.05$, $^{**}p < 0.01$, with Student's t test.

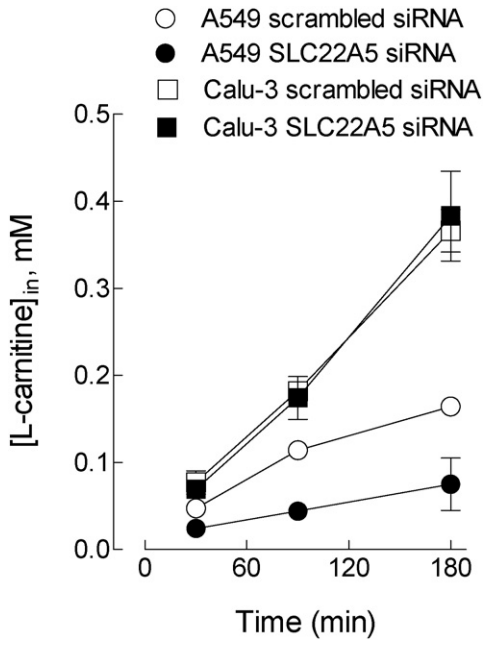

Fig. 9. *Time-dependent accumulation of L-carnitine in A549 and Calu-3 cells silenced with SLC22A5/OCTN2.* A549 and Calu-3 cells were transfected with scrambled or SLC22A5/OCTN2 siRNA for 96 h, as described in Materials and methods. Cells were then incubated for the indicated time in the transport buffer (see Materials and methods) containing L-[^{3}H]carnitine (30 μM; 2 μCi/ml). Each point represents the mean ± S.D. of four independent determinations. The experiments has been repeated three times with comparable results.

of carnitine transport by leucine and arginine, two amino acid substrates of ATB$^{0,+}$, as well as its chloride-dependence, identify the latter component with ATB$^{0,+}$. The K_m values for OCTN2 and ATB$^{0,+}$ obtained here are consistent with those reported for the corresponding transporters in other cell models [18,20]. The estimated kinetic parameters (see Table 1) indicate that the relative contribution of OCTN2 is prevalent at low concentrations of substrate, whereas that of the low-affinity component (ATB$^{0,+}$) becomes prevalent as carnitine concentration increases. In vivo, however, it is presumable that the intracellular content of carnitine is mainly determined by the activity of OCTN2 since ATB$^{0,+}$ activity, under physiological conditions, is likely inhibited by the presence of amino acids (such as leucine, glutamine, arginine), which can compete with carnitine.

The physiological significance of ATB$^{0,+}$ transporter in the lung is to promote an efficient protein clearance through the active reabsorption of amino acids, hence playing a role of critical importance under pathological conditions associated to protein accumulation in the airway spaces, such as acute respiratory distress syndrome (ARDS) or pulmonary alveolar phospholipoproteinosis (PAP) [40]. In our contribution we demonstrate that this transporter can operate also carnitine uptake, as already suggested by the group of Ganapathy [18]. The presence of this transporter in the cells appears of peculiar relevance in the pharmacological therapy of systemic primary carnitine deficiency (CDSP), an autosomal recessive disorder of carnitine transport caused by mutations in the SLC22A5 gene. In CDSP, functional defect of OCTN2 [1,16] leads to low serum carnitine levels resulting in defective fatty acid oxidation responsible for the clinical manifestations. Primary therapeutic treatment consists, hence, in the oral supplementation with levocarnitine (L-carnitine) which prevents the metabolic and myopathic manifestations of CDSP by maintaining normal plasma carnitine levels. It has been

previously postulated that when OCTN2 is genetically compromised, $ATB^{0,+}$ activity is essential for carnitine absorption [18]. Accordingly, here we find that when OCTN2 activity is impaired by gene silencing, Calu-3 cells, but not A549 that lacks $ATB^{0,+}$, can still accumulate high amounts of carnitine (Fig. 9), hence indicating that $ATB^{0,+}$ transporter can compensate for the lack of OCTN2 in supplying cells with extracellular carnitine. This appears to be of peculiar relevance for carnitine homeostasis in patients with genetic defects in OCTN2.

OCTN1 and OCTN2 transporters are highly homologous but have very different specificities [42]. OCTN1 has been reported to mediate carnitine transport even if with a very low affinity [13]. Conversely, the group of Longo N. previously reported that the human OCTN1 failed to cause any significant increase in L-carnitine transport in CHO cells transfected with chimeric OCTN1 transporter [42]. Our results are consistent with this latter finding, since no contribution of a low-affinity component to carnitine transport was detectable in A549 and BEAS-2B cells (Fig. 2), despite the evident expression of OCTN1 protein in these cells (see Fig. 3).

On the other hand, we demonstrate here that the preferential substrate of OCTN1, ergothioneine [10], inhibits carnitine transport in A549 and BEAS-2B cells when employed at high concentrations (1 mM, see Fig. 4). This effect is clearly due to the inhibitory effect of ET on OCTN2 since, when carnitine uptake was measured in the presence of betaine, the addition of ET halved betaine $I_{0.5}$ value, without affecting its I_{max}, hence demonstrating that the two compounds share the same transporter, OCTN2 (Fig. 5). This finding is further supported by the results obtained in CHO cells transfected with OCTN2, where ET inhibited carnitine uptake, even if only at the highest concentration (see Fig. 5, panel B). Our results demonstrate that ET cannot be considered a substrate of the sole OCTN1, since it can also interact with OCTN2, although with a low affinity.

Although the specific physiological role of OCTNs in the lung is not completely understood, the identification of these transporters and the substrates carried are of clinical importance because they can play a significant role in the delivery of cationic drugs such as bronchodilators. The work by Nakamura et al. provided the first evidence that OCTN2 contributes at least in part to the delivery of cationic anti-COPD drugs in BEAS-2B cells [43]; in the same cell model, Mo et al. reported that L-carnitine ester derivatives of prednisolone (PDSC) are transported by OCTN2 [44]. Moreover, also $ATB^{0,+}$ can be a potential delivery system for a wide variety of drugs: it has been reported that $ATB^{0,+}$ can transport antiviral drugs such as acyclovir and ganciclovir when they are covalently coupled to the side chain of amino acids [45], and recently, the same transporter is under preclinical investigation as a drug target for multiple approach in anticancer therapy. The group of Ganapathy identified this transporter as a novel and effective drug target for the treatment of estrogen receptor (ER)-positive breast cancer [46], and Muller et al. provided evidence for an $ATB^{0,+}$-selective PET probe which may help in evaluating $ATB^{0,+}$-targeting drug candidates *in vivo* in preclinical drug development programs [47]. Thus, since the airway epithelium represents a barrier which inhaled drugs must cross to reach targeted receptors in the underlying airway smooth muscle, a better understanding of the pulmonary transport mechanisms should provide information which can be used to develop more effective inhaled drugs for the treatment of pulmonary diseases. Whether differences among the expression and activity of carnitine transporters here observed in immortal, continuously growing cell lines, are due to the specific district of origin of the cells or to their malignant phenotype deserves to be further addressed. Anyway, it is noteworthy that the results presented here might be useful for the selection of cell models suitable for transport studies.

Conflict of interest

The authors declare no competing financial interests.

Transparency document

The Transparency document associated with this article can be found, in online version.

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