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Beneficial effects of L-carnitine in myoblastic C2C12 cells Interaction with zidovudine

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Abstract

L-Carnitine is a key molecule in the transfer of fatty acid across mitochondrial membranes. Bioavailable L-carnitine is either provided by an endogeneous biosynthesis or after intestinal absorption of dietary items containing L-carnitine. After intestinal absorption or hepatic biosynthesis, L-carnitine is transferred to organs whose metabolism is dependent upon fatty acid oxidation, such as skeletal muscle. To cross the muscle plasma membrane, there are several transporters involved. Among those transporters, OCTN2 is actually the only one to have been clearly characterized. Zidovudine is a commonly used inhibitor of human immunodeficiency virus (HIV) replication. Zidovudine has many side effects, including induction of myopathy characterized by a metabolic mitochondria dysfunction and a diminution of the muscle L-carnitine content. In this study, we described the characteristics of L-carnitine transport in C2C12 cells. We also demonstrated that zidovudine inhibited the L-carnitine transporter. This inhibition led to a significant reduction of the muscle cell growth. In C2C12 cells, the supplementation of L-carnitine prevented the effects of zidovudine and restored the normal cell growth.

Keywords: L-Carnitine; Zidovudine; C2C12; Transporter; Skeletal muscle; Lipid metabolism

1. Introduction

L-Carnitine is a small hydrophilic molecule of 162 Da derived from lysine and methionine. Inside the cell, L-carnitine interacts with acyl-CoA permitting their entry into the mitochondrial matrix. These acyl-CoA can enter in the β-oxidative pathway and then provide energy to the cell. L-Carnitine is clearly a key element in the oxidative pathway of long-chain fatty acids. L-Carnitine found in human body is either provided by food stuff, especially by meat and dairy products, or derived from an endogeneous synthesis. L-Carnitine coming from food is actively absorbed by intestine mucosa. This is a Na^+-dependent transport that appears to be efficient enough to allow up to 85% of dietary L-carnitine to be absorbed [1]. In rat, the biosynthesis is limited to hepatic cells, while, in human, kidney cells also participate.

Once synthesized or absorbed, L-carnitine is distributed to organs and tissues dependent upon fatty acid oxidation and especially muscle. Ninety to ninety-five percent of total L-carnitine is found in this tissue and the ratio between muscle and plasma L-carnitine concentration is around 50:1 [2]. The muscle is unable to synthesize L-carnitine, thus this molecule has to be imported from the blood stream. The import of L-carnitine across the muscle plasma membrane is done by at least one transporter. Among those, only one transporter has been clearly identified, it is Organic Cation Transporter New 2 (OCTN2) which belongs to the Organic Cation Transporter family [3]. A defect of this transporter leads to a myopathy, a cardiomyopathy and lipid metabolism disorders [4]. The presence of other transporters has been argued, however, these proteins have never been clearly described or isolated.

C2C12 cells are myoblastic cells commonly used as model for studying muscle cell growth and differentiation [5]. They exhibit most of the characteristics of normal

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Abbreviations: AIDS, Acquired Immuno Deficiency Syndrome; AZT/zidovudine, 3′-azido-3′deoxythymidine; OCT(N), Organic Cation Transporter (New); PBS, Phosphate buffer saline; THP, Trimethyl hydrazinium propionate.

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myoblastic cells and particularly the presence of an active L-carnitine transport activity.

3′-Azido-3′deoxythymidine (zidovudine or AZT) is a reference antiretroviral drug used in the treatment of Acquired Immuno Deficiency Syndrome (AIDS) patients. Once in the cell, zidovudine is phosphorylated and incorporated into viral DNA. Chemical modifications in the 3′-OH group of deoxyribose, which normally forms 3′–5′ phosphodiester bond in the DNA molecule prevent the addition of nucleotides [6]. This prevents DNA elongation and also inhibits viral reverse transcriptases [7–9].

Unfortunately, in AIDS therapy, the clinical efficiency of zidovudine is limited by its side effects. In zidovudine-treated patients, skeletal myopathy has been reported at a rate of 17% [8]. This skeletal toxic mitochondrial myopathy is characterized by muscle weakness, myalgia, ragged-red fibbers [10], cytochrome c oxidase deficiency [11], elevated blood lactate to pyruvate ratio [12]. A reduction of muscle L-carnitine within muscle fibers is also observed. The muscle mitochondrial dysfunction induced by zidovudine resulted in poor utilization of long-chain fatty acids, leading to the accumulation of lipids within muscle cells [4]. Most of these side effects seem to be related to a muscle mitochondrial DNA depletion [13], but not all of them.

In zidovudine-treated patients, a supplementation of L-carnitine has been shown to preserve the structure of mitochondria and to reduce the degeneration of myofibrils [14]. The effect of zidovudine on intracellular L-carnitine level cannot be fully explained by the depletion of mitochondrial DNA. In the present study, we characterized the normal properties of L-carnitine transport in C2C12 cells and the effect of zidovudine on L-carnitine import in these cells.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma. Tritiated L-carnitine (L-[methyl-^{3}H]Carnitine, specific activity 80 Ci/mmol) was obtained from Amersham Pharmacia Biotech. Culture medium, fetal bovine serum and other cell culture ingredients were purchased from Biomédia.

2.2. Cell culture

All experiments were done on C2C12 cells obtained from ATCC (through LGC Promochem, Teddington, UK). C2C12 cells are murine myoblastic cells capable to multiplication and differentiation while growing. These cells were cultured according to the recommendations of ATCC (i.e. in DMEM medium supplemented with 4.5 g/L glucose, 10% fetal bovine serum, 4 mM glutamine, 1.5 g/L $NaHCO_3$, 1 mM sodium pyruvate, penicillin 100 IU/mL, streptomycin 100 μg/mL, and amphotericin B 250 μg/mL) and grown at 37° in a humidified atmosphere of 5% CO_2. C2C12 cells were subcultured at 70% of confluence. Cells used in these experiments were at passage number 8.

2.3. Cell growth

Viable cells were numbered with trypan blue exclusion coloration after 24, 48, and 72 hr of treatment.

2.4. Determination of L-carnitine transporter activity

Cells were platted onto 12-well plates at the density of 25×10^4 cm^2. L-Carnitine uptake measurement was carried out at 37°. The incubation medium contained 135 mM NaCl (or 135 mM choline), 5 mM KCl and 20 mM Tris–HCl (pH 7.5). The medium contained 12.5 nM of radiolabeled L-carnitine and various concentrations of unlabeled L-carnitine. After a 45-min incubation, the medium was removed and the cells were washed four times with ice-cold phosphate buffer saline (PBS 1×). The C2C12 cells were scraped in 1 mL of phosphate buffer saline 1× and the L-carnitine transport was estimated by measuring the radioactivity in the sample.

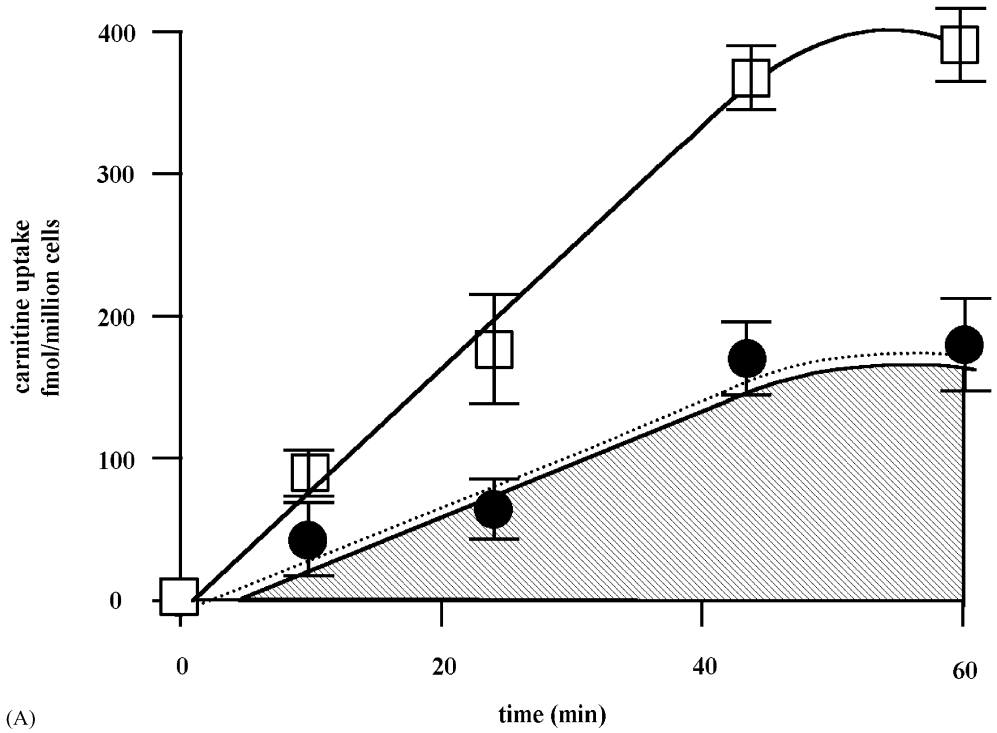
2.5. Statistics

Experimental conditions were performed at least in triplicate. The result of these is given as the mean ± SE. Statistical analysis were performed using the Student's test (Statistica). A value of $P < 0.05$ was interpreted to denote statistical significance.

3. Results

3.1. Characterization of L-carnitine transport in C2C12 cells

The normal course of the uptake of L-carnitine in C2C12 cells showed that this transport activity is, at least, in part, inhibited in the absence of sodium ion (Fig. 1A). When an equimolar amount of choline was substituted for sodium ion, an inhibition was observed. L-Carnitine transport activity increased with time, linearly for at least 1 hr when Na^+ ion was present. When choline was used to replace sodium, basically 50% of L-carnitine uptake was still observed. This suggested the presence of two components for L-carnitine uptake: one Na^+-dependent and one Na^+-independent activity. In our experiments, the upper curve of Fig. 1A represented the sum of the Na^+-dependent and the Na^+-independent activities. If Na^+-independent activity is removed from the curve, the resulting activity represented the net Na^+-dependent activity.



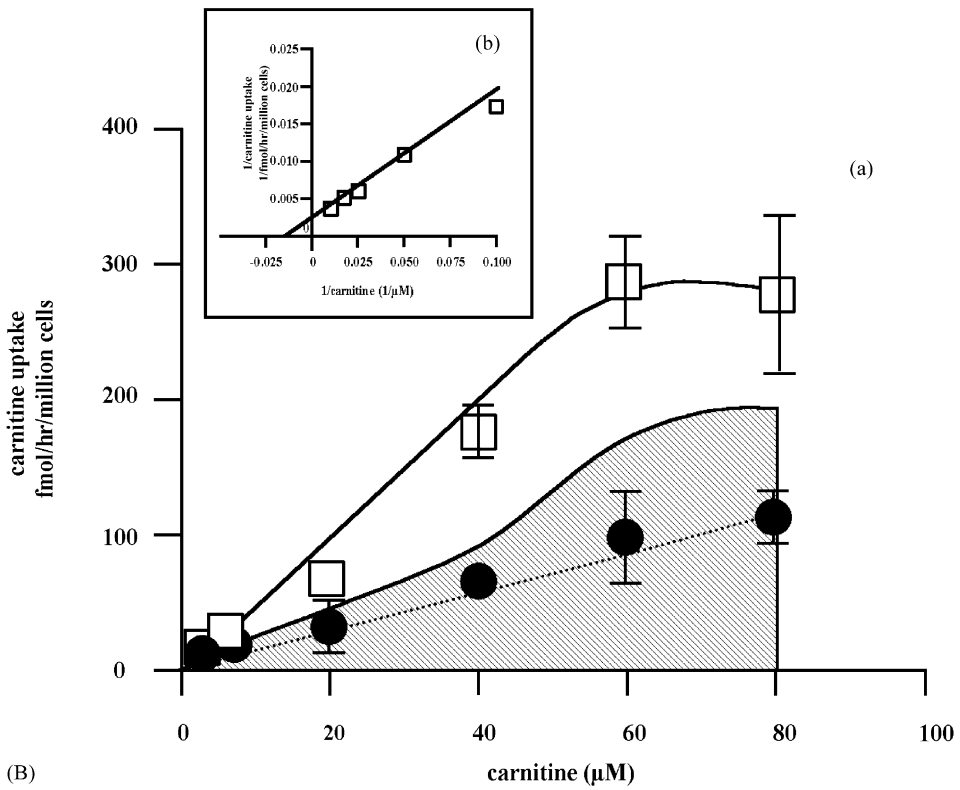


Fig. 1. (A) Normal time course of L-carnitine uptake by C2C12 cells. C2C12 cells were incubated (from 0 to 60 min) in a reaction mixture containing either 135 mM NaCl (□ and plain line) or 135 mM choline (● and dashed line). Transporter activity was determined as the amount of carnitine that crossed the membrane and expressed in fmol/million cells. Each experiment was done at least five times and the symbols represented the means and the SE. The Na^+-dependent component of L-carnitine uptake is denoted by the shaded curve. (B) Kinetics of L-carnitine transport into C2C12 cells. (a) C2C12 cells were incubated 45 min in a reaction mixture containing either 135 mM NaCl (□ and plain line) or 135 mM choline (● and dashed line) and various concentrations of carnitine (from 0 to 80 μM). Transporter activity was determined as the amount of carnitine that crossed the membrane and expressed in fmol/hr/million cells. Each experiment was done five times and the symbols represented the means and the SE. The Na^+-dependent component of L-carnitine uptake is denoted by the shaded curve. (b) Kinetics of the uptake was analyzed according to the Lineweaver–Burk representation. It allowed for determining K_m and V_{max} values.

3.2. Determination of apparent kinetics parameters for L-carnitine transport

Apparent K_m and V_{max} values were determined for L-carnitine uptake. These values were determined on entire C2C12 cells in the presence or the absence of extracellular sodium. In the presence of Na^+, the transport appeared as saturable and a apparent K_m value of 63.7 μM was found with a V_{max} of 384.6 fmol/hr/10^6 cells. In the absence of sodium, the transport was found to be linear (at least up to 80 μM of L-carnitine). The net Na^+-dependent L-carnitine transport can be deduced from these two curves. In this case, the Na^+-dependent L-carnitine transport was saturable, not the Na^+-independent L-carnitine transport activity (Fig. 1B). The kinetics parameters of the Na^+-dependent L-carnitine transport activity are a V_{max} of 193.3 fmol/hr/10^6 cells and a K_m of 40 μM.

3.3. Substrate specificity of L-carnitine uptake in C2C12 cells

The substrate specificity of L-carnitine transport was estimated by testing the effects of structural analogue compounds on C2C12 cells. These experiments were done in the absence and the presence of extracellular Na^+ ions. The tested compounds were D-carnitine, gamma-butyrobetaine, and trimethyl hydrazinium propionate (THP or also called mildronate). These L-carnitine-related molecules were used at a concentration of 500 μM. As shown in Fig. 2, in the presence of Na^+ ions, D-carnitine, gamma-butyrobetaine, and THP were potent inhibitors of L-carnitine transport. In the absence of Na^+ ions (i.e. when Na^+ ions were replaced by choline), it appeared that none of these compounds were able to inhibit the Na^+-independent L-carnitine transport.

3.4. Effect of zidovudine on L-carnitine transport activity in C2C12 cells

Zidovudine is known to depress L-carnitine content in muscle cells, however, the precise mechanism used by this nucleotide analogue remained unknown. We tested the effect of zidovudine on the kinetic parameters of L-carnitine transport in C2C12 cells. To clearly assess which component of L-carnitine uptake was involved, these experiments were done either in the presence of Na^+ ions or in the absence of these ions. As shown in Fig. 3, in the presence of Na^+ ions, the activity of L-carnitine transporter decreased as zidovudine concentration increased. In the same time, in the absence of Na^+ ions, the L-carnitine uptake by C2C12 cells remained unchanged. This clearly showed that only the Na^+-dependent component of L-carnitine transport was affected by this treatment in C2C12 cells. The inhibition was virtually complete for a concentration of zidovudine of 50 μM. From the dose–response curve given in Fig. 3, the IC_{50} value (concentration of the compound necessary to cause a 50% inhibition of the specific uptake of radiolabeled carnitine) was found to be 6 μM.

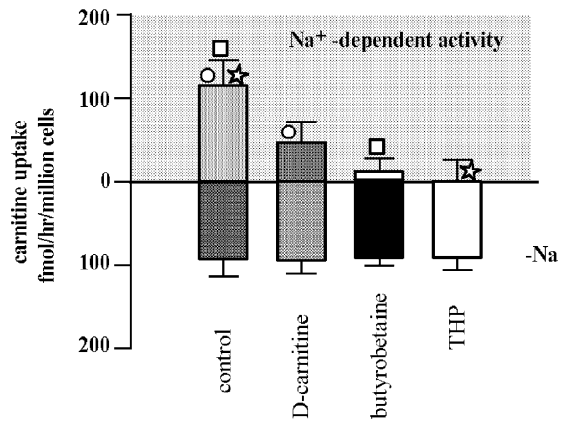


Fig. 2. Effect of D-carnitine, gamma-butyrobetaine and THP on the carnitine transport in C2C12 cells. C2C12 cells were incubated 45 min in a reaction mixture containing either 135 mM NaCl or 135 mM choline and 500 μM of the carnitine analogous. Transporter activity was determined as the amount of carnitine that cross the membrane and expressed in fmol/hr/million cells. Each experiment was done at least five times. The entire histogram represents the total transporter activity as found in the presence of Na^+ ions. The lower part of the histogram (below the horizontal black line) represents the L-carnitine transporter activity in the absence of Na^+ ions. The resulting Na^+-dependent L-carnitine activity is represented by the upper part of the histogram. Carnitine transport activity is expressed in fmol/hr/million cells. Means of three experiments are given as ±SE. The symbols (□, ○, and ☆) represent statistically significant differences (□: $P < 0.05$, ○: $P < 0.001$, ☆: $P < 0.001$).

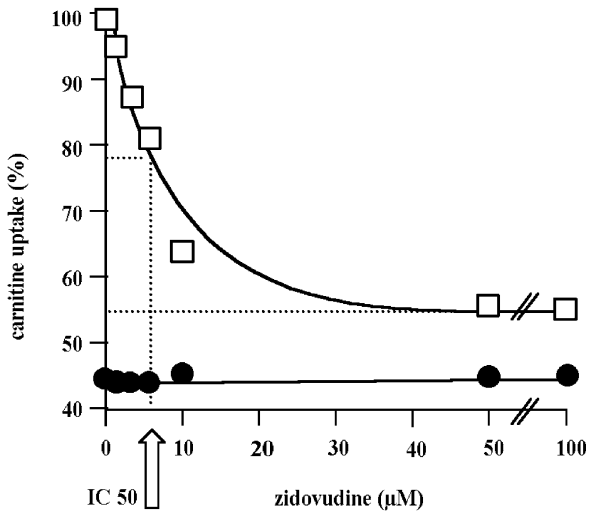


Fig. 3. Inhibition of L-carnitine uptake by C2C12 cells in the presence of zidovudine. C2C12 cells were incubated 45 min in a reaction mixture containing either 135 mM NaCl (□) or 135 mM choline (●) in the presence of various concentrations of zidovudine. Transporter activity was determined as the amount of carnitine that crossed the membrane and expressed in percentage of the maximal activity. Each experiment was done at least four times. The points on the curve represent the average of the percentages for each concentration. Only the Na^+-dependent activity was decreased in the presence of zidovudine. Using these data, we determined an IC_{50} for zidovudine inhibition of L-carnitine uptake in C2C12 cells, a value of 6 μM was found.

3.5. Measurement of the cell growth in the presence of zidovudine and L-carnitine

C2C12 are myoblastic cells whose growth is close to the development of human muscle cells. We and other previously reported that the growth of these cells was under the control of L-carnitine bioavailability [15]. Since zidovudine inhibited L-carnitine transport we investigated if zidovudine could repress C2C12 development through an inhibition of L-carnitine transport. This molecule was added to the culture at a concentration of 1 mM in the regular culture medium, i.e. without added L-carnitine. This value of 1 mM was chosen because, in our conditions, it was the concentration that allows the highest inhibition of L-carnitine transport without inducing cell death. After 24, 48, and 72 hr of treatment, C2C12 cells were numbered. At this concentration of zidovudine and after 24 hr of treatment, the cell growth was decreased by 27.6% ($P < 0.05$). After 48 and 72 hr of treatment, the inhibition of the growth represented, respectively, 26.5% ($P < 0.005$) and 41.0% ($P < 0.005$) compared to control cell growth (Fig. 4).

To understand the interrelation between carnitine and zidovudine on L-carnitine transport, this uptake of L-carnitine was examined on C2C12 cells in the presence of zidovudine and in the presence of L-carnitine concentrations ranging from 20 to 60 μM. The C2C12 were cultured during 24, 48, and 72 hr. After 24 hr of treatment, the presence of 60 μM of L-carnitine restored the regular C2C12 growth that has been initially inhibited by zidovudine (Fig. 4). This result was also observed at 48 and 72 hr of treatment. These data suggested that L-carnitine prevented the inhibition of C2C12 cell growth observed in the presence of zidovudine.

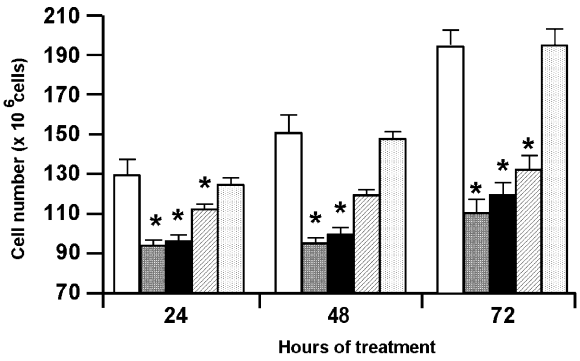


Fig. 4. Alteration of C2C12 cell growth in the presence of zidovudine. C2C12 cells were cultured under several conditions for times varying from 1 to 3 days. After this treatment, viable cells were counted. The white histogram represents cells cultured in the presence of a control medium and can be considered as control cells, the dark gray histogram represents cells cultured in the presence of a control medium supplemented with 1 mM of zidovudine, the black histogram represents cells cultured in the presence of 1 mM of zidovudine and 20 μM of carnitine, the shaded histogram represents cells cultured in the presence of 1 mM of zidovudine and 40 μM of carnitine, the grey histogram represents cells cultured in the presence of 1 mM of zidovudine and 60 μM of carnitine. Each histogram represents the average of five experiments ± SE. The asterisk indicates a significant difference between the considered data and the control values.

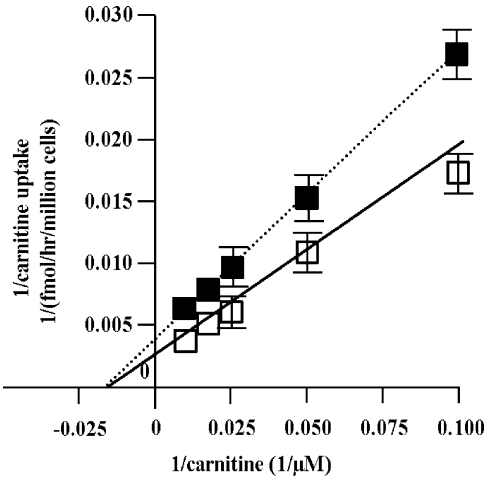


Fig. 5. Effect of zidovudine on carnitine transporter activity in C2C12 cells. C2C12 cells were incubated in the presence of various concentrations of L-carnitine (1 to 100 μM; □). Zidovudine was added to a concentration of 1 mM (■). K_m and V_{max} were estimated by the double transformation of Lineweaver–Burk. Each symbol represents the means and the SE of at least three experiments.

3.5.1. Interaction of zidovudine with L-carnitine transport

Zidovudine is a molecule exhibiting various toxic effects. To investigate the inhibition process that this drug exerted on L-carnitine uptake, we determined the kinetics parameters of zidovudine inhibition on L-carnitine uptake by C2C12 cells. As shown in Fig. 5, the V_{max} for the L-carnitine transport decreased in the presence of zidovudine (270.0 fmol/hr/10^6 cells) while the K_m remained unchanged. Analysis of these data provided evidence that zidovudine was a noncompetitive inhibitor of L-carnitine transport.

4. Discussion

Analysis of L-carnitine transport activity in C2C12 showed the presence of two components: one Na^+-dependent and another Na^+-independent that exhibited different kinetics characteritics. The Na^+-dependent component of the transport appeared to be saturable while the Na^+ independent part of this transport activity remained linear while L-carnitine concentrations increased. The Na^+ dependent component was shown to be inhibited by L-carnitine analogue and also by zidovudine (see below). It may be hypothesized that the Na^+-dependent L-carnitine transport represents the specific and controlled activity. The Na^+-independent component would represent a simple diffusion system or a low affinity transporter carrying L-carnitine among other compounds.

The presence of two components in L-carnitine uptake has also been described in the mammary tissue [16]. In this

tissue, 3–4 days *post partum*, it can be predicted from the kinetic data that, under physiological conditions, only 20% of L-carnitine uptake is done *via* the Na^+-independent component. In rodent and human muscle cells, it has been reported that a dual system for L-carnitine transport, one with low affinity for high concentrations (>60 μM) of L-carnitine and another one with high affinity, allows the transport of low concentrations of L-carnitine (<60 μM) [15]. In our experiments using C2C12 cells and in the range of L-carnitine concentrations we used, i.e. from 0 to 80 μM, we did not observe such a dual system. This may be due to the clonal origin of C2C12 cells or to the carnitine concentrations used in our experiments at which only the high affinity part is likely to be apparent.

Zidovudine inhibited the L-carnitine transporter in the myoblastic cells C2C12. Our data also showed that this inhibition was only shown when Na^+ ions were present. Zidovudine induced a noncompetitive inhibition of L-carnitine uptake and suggested that the molecule of zidovudine interacted directly with the L-carnitine transporter protein. This finding enforced the rational for a potential use of L-carnitine supplementation for patients treated with zidovudine.

In AIDS patients, a long-term treatment with zidovudine induces a mitochondrial-like myopathy. In these patients, a significant reduction of L-carnitine was found in the muscle and an accumulation of lipid droplets within the muscle fibers was observed [4], leading to the muscle pathology. Our findings strongly suggest that this reduction of intracellular L-carnitine is due to the inhibition of the L-carnitine transporter by zidovudine. It also appears that the Na^+-independent component for L-carnitine transport is probably not capable of compensating the lack of Na^+-dependent activity under physiological concentrations of carnitine.

On cultured human muscle cells, zidovudine induced a dose-related decrease of cell proliferation and differentiation [17]. Semino-Mora *et al.* [14] demonstrated that L-carnitine prevented the human myotubes from AZT-associated destruction, preserved the structure and the volume of mitochondria and prevented the accumulation of lipids. In our study, we showed that the inhibition of L-carnitine transport by zidovudine led to a reduction of the muscle C2C12 growth. This inhibition may be reversed by supplemental L-carnitine. It should also be mentioned that most of the effects of zidovudine, we described in this paper, were found for classical concentrations of zidovudine in treated patients. For AIDS patients, the dosage for zidovudine is classically 200 mg, six times a day. With a half life of 1 hr, this makes circulating concentrations of zidovudine ranging from 3 to 50 μM. The IC_{50} observed for the inhibition of L-carnitine was found to be 6 μM.

Our conclusions are: (i) that a zidovudine treatment led not to only a mitochondrial DNA depletion but also reduced the import of L-carnitine into the muscle cells and (ii) that the rational for a supplementation of L-carnitine in zidovudine-treated patients is increased.

Acknowledgments

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