European Journal of Pharmacology 682 (2012) 37–42

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and Cellular Pharmacology

Thiamine is a substrate of organic cation transporters in Caco-2 cells

Clara Lemos [a,b], Ana Faria [a,c], Manuela Meireles [a], Fátima Martel [a], Rosário Monteiro [a], Conceição Calhau [a,*]

[a] Department of Biochemistry (U38-FCT), Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal
[b] Max-Delbrück-Center for Molecular Medicine (MDC), Berlin, Germany
[c] Chemistry Investigation Centre (CIQ), Department of Chemistry, Faculty of Sciences, University of Porto, 4169-007 Porto, Portugal

ARTICLE INFO

Article history:
Received 1 July 2011
Received in revised form 7 February 2012
Accepted 15 February 2012
Available online 24 February 2012

Keywords:
Caco-2 cell
Organic cation
Uptake
Thiamine

ABSTRACT

The aim of this study was to characterize the intestinal absorption of thiamine, by investigating the hypothesis of an involvement of Organic Cation Transporter (OCT) family members in this process. [^{3}H]-T$^+$ uptake was found to be: 1) time-dependent, 2) Na^+- and Cl^--dependent, 3) pH-dependent, with uptake increasing with a decrease in extracellular pH and decreasing with a decrease in intracellular pH, 4) inhibited by amiloride, 5) inhibited by the thiamine structural analogues oxythiamine and amprolium, 6) inhibited by the unrelated organic cations MPP^+, clonidine, dopamine, serotonin, 7) inhibited by the OCT inhibitors decynium22 and progesterone. Moreover, the dependence of [^{3}H]-T$^+$ uptake on phosphorylation/dephosphorylation mechanisms was also investigated and [^{3}H]-T$^+$ uptake was found to be reduced by PKA activation and protein tyrosine phosphatase and alkaline phosphatase inhibition. In conclusion, our results are compatible with the possibility of thiamine being transported not only by ThTr1 and/or ThTr2, but also by members of the OCT family of transporters (most probably OCT1 and/or OCT3), thus sharing the same transporters with several other organic cations at the small intestinal level.

1. Introduction

Thiamine (vitamin B_1), a water-soluble micronutrient, is essential for normal cellular functions and growth. It is a precursor of thiamine pyrophosphate which plays a critical role in normal carbohydrate metabolism where it participates in the decarboxylation of pyruvic and α-ketoglutamic acids, and in the utilization of pentose in the hexose monophosphate shunt (Rindi and Laforenza, 2000).

Thiamine deficiency in humans leads to a variety of clinical abnormalities including neurological and cardiovascular disorders (Kumar, 2010; Morley, 2010; Soukoulis et al., 2009; Wooley, 2008). Thiamine deficiency represents a significant nutritional problem in developing countries. This is not an exclusive problem in these countries, as it is also found in affluent countries as a clinically significant problem in individuals with chronic alcoholism (Koike and Sobue, 2006; Subramanya et al.,; Tallaksen et al., 1992), diabetes (Saito et al., 1987; Thornalley et al., 2007), in patients with renal and intestinal diseases (Frank et al., 2000), and in the elderly (Fabian and Elmadfa, 2008; Lengyel et al., 2008), despite an average daily intake of this vitamin that exceeds the recommended requirements (Olsen et al., 2009).

Humans and other mammals cannot synthesize thiamine and thus must obtain this vitamin from exogenous sources via intestinal absorption. Thus, the intestine plays a critical role in regulating body thiamine homeostasis and understanding the mechanism of intestinal thiamine absorption process is of significant nutritional importance.

Chemically, thiamine is an organic cation (quaternary ammonium compound) with a high molecular weight. Biological membranes prevent transmembrane diffusion of the majority of organic molecules that bear net charges at physiological pH, and so membrane-bound transport systems are generally involved in the absorption, distribution, and elimination of these compounds. The first thiamine transporter (ThTr1, SLC19A2) was cloned in 1999 in yeast. Mutations in the human SLC19A2 gene are responsible for thiamine-responsive megaloblastic anemia (Rogers Syndrome) (Ganapathy et al., 2004). However, these patients do not present the characteristic cardiovascular and/or neurological symptoms (known as beriberi) seen in nutritional thiamine deficiency (Ganapathy et al., 2004), and have normal thiamine plasmatic levels, which suggest that, besides ThTr1, other transporter(s) are also involved in the intestinal transport of thiamine. Accordingly, a second high-affinity transporter was described for thiamine (ThTr2; SLC19A3). This transporter is expressed in different tissues, including intestine (Said et al., 2004).

Since thiamine is an organic cation, organic cation transporters (OCTs; members of the SLC22 family), which are polyspecific transporters of organic cations, may also contribute for global thiamine transport. At the intestinal level, two distinct OCTs are known to be functionally present: OCT1 (Martel et al., 2000) and OCT3 (also known as the extraneuronal monoamine transporter, EMT) (Martel et al., 2000, 2001).

For studies on the intestinal uptake of thiamine, Caco-2 cells, an enterocyte-like cell line derived from a human colonic adenocarcinoma,

* Corresponding author at: Department of Biochemistry, Faculty of Medicine, University of Porto, Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal. Tel./fax: +351 22 5513624.
E-mail address: ccalhau@med.up.pt (C. Calhau).

0014-2999/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2012.02.028

were used as an intestinal epithelial model. This human intestinal epithelial cell line forms confluent monolayers of well-differentiated enterocyte-like cells with the functional properties of transporting epithelia (Delie and Rubas, 1997; Yee, 1997).

2. Materials and methods

2.1. Materials

[^{3}H]-thiamine (specific activity 10 Ci mmol^{-1}; American Radiolabeled Chemicals Inc., St. Louis, MO, USA); DMSO, Triton X-100 (Merck, Darmstadt, Germany); tetraethylammonium bromide (TEA), cimetidine, choline chloride, N-methylnicotinamide chloride (NMN), corticosterone, decynium22 (1,1′-diethyl-2,2′-cyanine iodide), dibutyryl cAMP sodium salt, histamine dihydrochloride, β-estradiol, pyridoxal 5′-phosphate, HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), amiloride hydrochloride, amprolium hydrochloride, lithium chloride, clonidine hydrochloride, dopamine hydrochloride, guanidine hydrochloride, levamisole hydrochloride, oxythiamine hydrochloride, thiamine pyrophosphate, MEM (Minimal Essential Medium), progesterone, desipramine, fluoxetine, serotonin (5-HT), sodium orthovanadate, penicillin/streptomycin solution, riboflavin, Tris (tris-(hydroxymethyl)-aminomethane hydrochloride), trypsin-EDTA solution, fetal bovine serum (Sigma, St. Louis, MO, USA); cyanine863 (quinolinium,2-[(3,6-dimethyl-2-phenyl-4(3H)-pyrimidinylidene)methyl]-1-ethyl-chloride) (ICN Pharmaceuticals, Costa Mesa, CAL, USA).

2.2. Cell and culture conditions

The Caco-2 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and was used between passage number 14–74. Caco-2 cells (ATCC 37-HTB) were maintained in a humidified atmosphere of 5% CO_2–95% air and were grown in Minimum Essential Medium (Sigma, St. Louis, MO, USA) supplemented with 15% fetal bovine serum, 25 mM HEPES, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B. Culture medium was changed every 2 to 3 days and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 5 min, 37 °C), split 1:3, and subcultured in plastic culture dishes (21 cm^2; ∅ 60 mm; Corning Costar, Corning, NY). For the experiments, the Caco-2 cells were seeded on 24-well plastic cell culture clusters (2 cm^2; ∅ 16 mm; Corning Costar). For 24 h before the experiment, the cell medium was free of fetal bovine serum. Uptake and enzymatic studies were generally performed 9–20 days after the cells formed a monolayer.

2.3. Cellular uptake of [^{3}H]-T$^+$ by Caco-2 cells

[^{3}H]-thiamine ([^{3}H]-T$^+$) uptake experiments were performed in Hanks' medium with the following composition (in mM): 137 NaCl, 5 KCl, 0.8 $MgSO_4$, 1.0 $MgCl_2$, 0.33 Na_2HPO4, 0.44 KH_2PO_4, 0.25 $CaCl_2$, 0.15 Tris–HCl, and 1.0 sodium butyrate, pH 7.4. These studies were performed in Caco-2 cells cultured in plastic supports, [^{3}H]-T$^+$ being applied to the luminal cell border.

Initially, the growth medium was aspirated and the cells were washed with Hanks' medium at 37 °C; then the cell monolayers were preincubated for 20 min in Hanks' medium at 37 °C. Uptake was initiated by the addition of 250 μl medium at 37 °C containing 100 or 400 nM ^{3}H-T$^+$. After 3 min, incubation was stopped by placing the cells on ice and rinsing them with 500 μl ice-cold Hanks' medium. The cells were then solubilized with 300 μl 0.1% (v/v) Triton X-100 (in 5 mM Tris–HCl, pH 7.4), and placed at 37 °C overnight. Radioactivity in the cells was measured by liquid scintillation counting.

Effect of drugs: drugs to be tested were present during both the preincubation and incubation periods. Controls were performed in the presence of 0.1% of the solvent.

Effect of pH or ionic composition of the external medium: to determine the influence of external medium pH on the uptake of [^{3}H$^+$]-T$^+$, cells were incubated in pH-variable media (5.0–8.5). To study Na^+- and Cl^--dependence of the uptake of [^{3}H$^+$]-T$^+$, cells were preincubated and incubated in NaCl-free medium. NaCl was isotonically replaced by lithium chloride, choline chloride, potassium chloride or sodium fluoride.

2.4. Protein quantification

The protein content of cells was determined by the method of Bradford (1976), with human serum albumin as standard.

2.5. Calculations and statistics

Arithmetic means are given with S.E.M. Statistical significance of the difference between various groups was evaluated by one-way analysis of variance (ANOVA test) followed by the Bonferroni test. For comparison between two groups, Student's *t*-test was used. Differences were considered to be significant when $P<0.05$.

3. Results

3.1. Characterization of thiamine ([^{3}H]-T$^+$) uptake by Caco-2 cells

Cellular uptake of [^{3}H]-T$^+$ was determined using Caco-2 cells as a model of intestinal epithelia. In the first series of experiments, we verified that cells took up [^{3}H]-T$^+$ in a time-dependent way, and that uptake of [^{3}H]-T$^+$ was linear with time for up to 5 min of incubation. So, all the subsequent experiments were performed using a 3 min incubation period, in the presence or absence of compounds, in order to determine initial rates of uptake (Fig. 1).

3.2. pH-dependence and amiloride effect

The effect of extracellular medium pH on [^{3}H]-T$^+$ uptake was examined by varying the pH of the preincubation and incubation media over the range between 5 and 8.5. Acidification of the extracellular media increased [^{3}H]-T$^+$ uptake, showing thus the pH-dependency of this transport (Fig. 2A).

The effect of intracellular acidification was next studied. For this, cell acidification was induced on confluent monolayers of Caco-2 cells, which were incubated for 30 min in Hanks buffer with NH_4Cl (in replacement of NaCl). This was followed by removal of the media and incubation for 3 min with 400 nM of [^{3}H]-T$^+$ in media where NaCl was replaced by KCl. For control, cells were incubated for 30 min in buffer that had KCl in replacement of NaCl. We observed a significant inhibition of [^{3}H]-T$^+$ uptake in NH_4Cl-treated cells, when compared to control cells (Fig. 2B). Finally, inhibition of Na^+/H^+

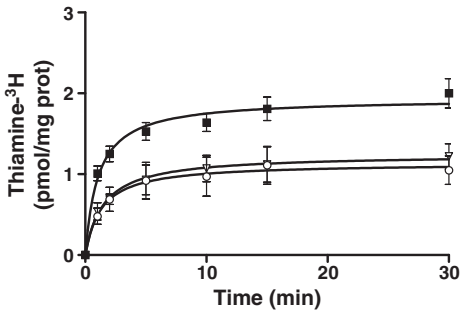

Fig. 1. Time-course of [^{3}H]-T$^+$ uptake by Caco-2 cells. Cells were preincubated for 20 min and then incubated with thiamine-^{3}H 100 nM at 37 °C in the absence (■; $n=4$) or presence of thiamine 100 μM (□; $n=2$) or amprolium 100 μM (▽; $n=2$). Data are shown as arithmetic means ± S.E.M.

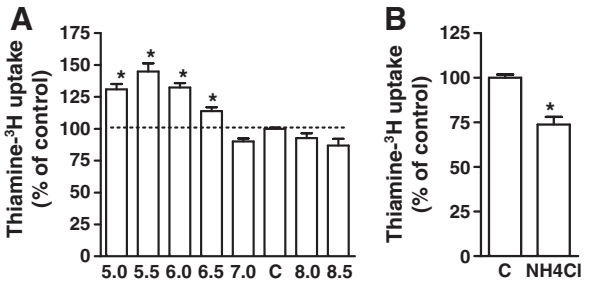

Fig. 2. A—Effect of extracellular pH (5 to 8.5 ($n=6$–10) on [^{3}H]-T$^+$ uptake by Caco-2 cells. Cells were preincubated for 20 min and then incubated at 37 °C with [^{3}H]-T$^+$ (400 nM) for 3 min. B—Effect of intracellular acidification ($n=6$) on [^{3}H]-T$^+$ uptake by Caco-2 cells. Cells were preincubated for 30 min in the absence (control) or presence of NH_4Cl and then incubated in the presence of KCl at 37 °C with [^{3}H]-T$^+$ (400 nM) for 3 min. *$P<0.05$ vs control.

exchanger with amiloride (1 mM) resulted in a significant decrease of [^{3}H]-T$^+$ uptake (47 ± 7% of control; $n=4$).

3.3. Potential-, sodium- and chloride-dependence

Replacement of NaCl by LiCl or KCl in both the preincubation and incubation media did not induced any significant changes on [^{3}H]-T$^+$ uptake (92 ± 5% and 108 ± 10% of control, respectively; $n=17$ and $n=10$). However, when NaCl was replaced by choline chloride (CoCl), there was a significant decrease of [^{3}H]-T$^+$ uptake. The use of NaF had a tendency to reduce the uptake of this vitamin (Table 1).

3.4. Effect of thiamine and thiamine analogs

Oxythiamine and amprolium significant decreased [^{3}H]-T$^+$ cellular uptake in a concentration-dependent manner (Fig. 1 and Table 1). Non-labeled thiamine also led to a significant and concentration-dependent reduction of its own transport (Fig. 1 and Table 1).

Table 1
Effect of several compounds on [^{3}H]-T$^+$ uptake by Caco-2 cells. Cells were preincubated for 20 min and then incubated at 37 °C with [^{3}H]-T$^+$ (400 nM) for 3 min in the absence (control; C) or presence of these compounds. Data are shown as pmol of [^{3}H]-T$^+$/mg prot/3 min. [a]$P<0.05$ vs control.

Compound	^{3}[H]-Thiamine uptake (pmol/mg prot/3 min)
Control	5.79 ± 0.47
CoCl	2.54 ± 0.41[a]
LiCl	5.35 ± 0.45
KCl	6.24 ± 1.1
NaF	4.94 ± 0.53
Control (H_2O)	7.00 ± 0.38
Oxythiamine 10 μM	5.49 ± 0.1[a]
Oxythiamine 100 μM	5.17 ± 0.1[a]
Oxythiamine 500 μM	4.06 ± 0.42[a]
Amprolium 10 μM	4.31 ± 0.71[a]
Amprolium 100 μM	3.67 ± 0.45[a]
Amprolium 500 μM	3.78 ± 0.42[a]
Thiamine 10 μM	4.89 ± 0.49[a]
Thiamine 100 μM	4.28 ± 0.17[a]
Thiamine 500 μM	4.13 ± 0.34[a]
Dopamine 100 μM	8.29 ± 0.22[a]
Clonidine 100 μM	5.72 ± 0.33[a]
MPP$^+$ 100 μM	5.12 ± 0.32[a]
Vanadate 100 μM	6.15 ± 0.52[a]
Levamisol 500 μM	3.92 ± 0.51[a]
Control (EtOH)	6.06 ± 0.36
D22 5 μM	4.48 ± 0.32[a]
D22 50 μM	4.04 ± 0.56[a]
Progesterone 100 μM	5.14 ± 0.45[a]
Control (DMSO)	7.06 ± 0.37
DB-AMPc 2.5 mM	6.01 ± 0.75[a]

3.5. Effect of OCTs modulators

The effect of several OCTs (organic cation transporters) modulators (substrates/inhibitors) on [^{3}H]-T$^+$ uptake in Caco-2 cells was next investigated. Clonidine (100 μM), MPP$^+$ (100 μM), D22 (5 and 50 μM) and progesterone (100 μM) were able to significantly inhibit [^{3}H]-T$^+$ uptake on Caco-2 cells (Table 1).

Other OCTs inhibitors tested, such as histamine (500 μM), cimetidine (100 μM), guanidine (100 and 500 μM), tetraethylammonium (TEA; 100 μM), choline (100 μM and 1 mM), NMN (100 μM), corticosterone (270 μM) and cyanine863 (1 μM), did not show any effect upon [^{3}H]-T$^+$ uptake by these cells (data not shown). Curiously, dopamine (100 μM) significantly increased [^{3}H]-T$^+$ uptake (Table 1).

3.6. Effect of phosphatases inhibitors

Two phosphatases inhibitors were tested, levamisole (500 μM) and vanadate (100 μM), and there was a significant inhibition of [^{3}H]-T$^+$ cellular uptake in the presence of these compounds, when compared to control. Levamisole (56 ± 5% of control) had a more pronounced effect than vanadate (88 ± 3% of control) (Table 1).

3.7. Effect of DB-AMPc

Cells were treated for 20 min with DB-AMPc (2.5 mM), an analogue of AMPc that increases intracellular levels of AMPc. This treatment significantly decreased [^{3}H]-T$^+$ uptake, which suggests a possible involvement of protein kinase A (PKA) in the regulation of the apical transport of thiamine on Caco-2 cells (Table 1).

3.8. Effect of SERT modulators

Serotonin (5-HT) and two specific inhibitors of the serotonin transporter SERT (fluoxetine and desipramine) were tested in this model. Interestingly enough, [^{3}H]-T$^+$ uptake was significantly decreased in the presence of serotonin, but the SERT inhibitors have no effect on this transport (Fig. 3).

4. Discussion

The intestinal transport of thiamine is pharmacologically poorly characterized. The absorption of this vitamin is made primarily in intestine, and due to the fact that thiamine is an organic cation, it needs a transporter in the plasma membrane.

Previous studies with brush border membrane vesicles (BBMVs) revealed that thiamine uptake by enterocytes is a Na$^+$-independent, pH-dependent, amiloride-sensitive, electroneutral carrier-mediated

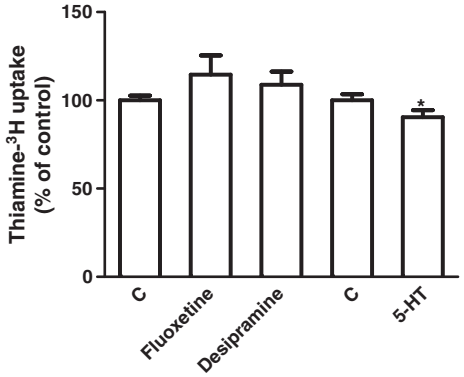

Fig. 3. – Effect of fluoxetine (10 μM), desipramine (10 μM) and serotonin (5-HT; 100 μM) on [^{3}H]-T$^+$ uptake by Caco-2 cells. Cells were preincubated for 20 min and then incubated at 37 °C with [^{3}H]-T$^+$ (400 nM) for 3 min in the absence (control; C) or presence of this compound. Data are shown as % of control. *$P<0.05$ vs control.

mechanism inhibited by thiamine structural analogs such as amprolium and oxythiamine and corresponding to a thiamine/H^+ antiport mechanism (Dudeja et al., 2001, 2003).

Moreover, some authors used Caco-2 cells and found that apical uptake of thiamine by these cells was also energy-, temperature- and pH-dependent and sodium-independent. It involves a saturable mechanism, being inhibited by thiamine structural analogs such as amprolium and oxythiamine, but not by other organic cations such as choline, TEA and NMN. Again, the results were compatible with the involvement of a thiamine/H^+ antiport mechanism (Said et al., 1999). Because both ThTr-1 and ThTr-2 function as thiamine/H^+ antiporters (Diaz et al., 1999; Labay et al., 1999; Rajgopal et al., 2001), both these transporters were suggested to be involved in the apical uptake of thiamine in Caco-2 cells and human intestine. Later work clearly showed that indeed this was the case (Said et al., 2004).

In what concerns thiamine efflux from enterocyte, studies using basolateral vesicles showed that this is sodium-dependent and inhibited by structural analogs of thiamine. In addition, an ATPase-Na^+-K^+ seems to be involved on basolateral transport of thiamine (Rindi and Laforenza, 2000).

In the present work, apical uptake of [^{3}H]-T^+ by Caco-2 cells was shown to be sodium- and potential-independent, since isosmolar substitution of extracellular NaCl by LiCl or KCl did not significantly change transport. Inhibition by choline chloride does not seem enough to support the conclusion that this transport is sodium- dependent. Since choline is an organic cation, we hypothesize that thiamine uptake inhibition is a consequence of competition between choline and thiamine for the same transporter. In the presence of lower choline concentrations (100 μM and 1 mM), however, we did not observe any change on [^{3}H]-T^+ transport (data not shown), which is in agreement with Said et al. (Said et al., 1999). Interestingly enough, Martel et al. (2001) described a similar effect of choline upon OCT3-mediated transport of the organic cation MPP^+ transport. According to these authors, high concentrations of choline (125 mM) significantly inhibited this transport, but lower concentrations had no effect.

We also found intestinal uptake of [^{3}H]-T^+ to be chloride-independent, as replacement of NaCl by NaF did not produce any effect.

Still according to the present study, thiamine transport in Caco-2 cells was inhibited, in a concentration-dependent manner, by the thiamine structural analogs amprolium and oxythiamine. These results suggest the involvement of at least one of the two transporters cloned for thiamine, ThTr1 and/or ThTr2, in thiamine uptake by Caco-2 cells.

However, the pH-dependence experiments point to the conclusion that, in Caco-2 cells, apical absorption of thiamine occurs through a thiamine/H^+ cotransport. These results can be summarized as follows: (i) acidification of extracellular media increased thiamine uptake and alkalinization decreased it; (ii) intracellular acidification resulted in inhibition of uptake; (iii) treatment of the cells with amiloride, an inhibitor of Na^+/H^+ exchanger present on apical membrane of enterocytes, caused a significant reduction of thiamine uptake. Taken together, the above results support the hypothesis of an involvement of a T^+/H^+ cotransport in thiamine uptake by Caco-2 cells, and not an T^+/H^+ antiport, as previously suggested (Said et al., 1999). Of note, Said et al. (1999) also confirmed a decrease in the uptake of thiamine by Caco-2 cells in the presence of amiloride. Since amiloride is a diuretic used on clinical practice, it seems important to understand the nutritional significance of this interaction, especially on patients chronically treated with this drug. It has been described that chronic use of diuretics can lead to a thiamine deficiency, which could be explained by these results. Thus, thiamine supplementation of patients subjected to chronic treatment with these drugs could be necessary.

As mentioned above, our pH-dependence results point to the involvement of a transporter distinct from either ThTr1 or ThTr2 in the apical uptake of thiamine by Caco-2 cells. A study of thiamine transport in placental trophoblastic cells (BeWo cells) also suggested the involvement of other transporters, besides ThTr1 and ThTr2, on thiamine cellular uptake. Authors concluded that SERT, a highly selective serotonin plasmalemmal transporter, could be involved in uptake of this vitamin at the placental level (Keating et al., 2006). However, in our work, the lack of effect of SERT inhibitors upon thiamine uptake shows that this transporter is most probably not involved in thiamine uptake by Caco-2 cells. If that is the case, then the significant decrease of thiamine uptake caused by serotonin could indicate that these two substances share another transporter. Serotonin transport by a member of the SLC22 family (OCT2) has already been described (Busch et al., 1998). Also, thiamine was found to inhibit serotonin transport in placental cells (Keating et al., 2004), reinforcing the theory that these two substances can, at least in part, share the same transporter.

Several endogenous substances and drugs from different therapeutics classes (e.g. neurotransmitters, some vitamins, antihistamines) are organic cations. At the intestinal epithelial level, they are thought to be transported by two different transporters belonging to the SLC22 transporter family: OCT1 and OCT3 (Martel et al., 2000, 2001). According to these and other authors, OCT3 is probably the main transporter involved on the apical uptake of organic cations (review by Koepsell et al., 2007). Since thiamine is absorbed in the intestinal lumen as an organic cation, the interference of organic cation transporters was hypothesized and investigated.

The organic cations TEA, choline and NMN, described as substrates/inhibitors of OCTs, did not have any effect on thiamine transport, which is in agreement with other authors (e.g. (Laforenza et al., 1998; Said et al., 1999)). Moreover, other OCTs substrates/inhibitors tested (namely histamine, cimetidine, guanidine, corticosterone and cianine 863), also did not interfere with the studied transport. On the contrary, some other OCT substrates/inhibitors (namely, clonidine, decynium22 and MPP^+) significantly inhibited thiamine transport in Caco-2 cells. Because MPP^+ is an excellent model substrate for OCT1, OCT2 and OCT3 but not for other members of the SLC22 family (Grundemann et al., 1999), these results suggest the involvement of one of these transporters on thiamine uptake. Curiously, dopamine, an organic cation known to be an OCT1, OCT2 and OCT3 substrate/inhibitor (Koepsell et al., 2007), significantly increased this transport. This could be due to a transtimulation phenomenon, thus indicating that an organic cation: organic cation antiporter mechanism may be operating.

Another study on Caco-2 cells showed that thiamine was able to decrease MPP^+ uptake on these cells, and the involvement of OCT3 on thiamine uptake by these cells was suggested (Calhau et al., 2003). Further, it was also concluded that this effect may be direct (as thiamine may be an OCT3 substrate) or indirect (through activation of ecto-ALP) (Calhau et al., 2003).

The modest inhibition of ^{3}H-thiamine uptake observed when a high concentration of cold thiamine (100 μM) was tested could result from: (1) the low affinity of hOCTs in relation to thiamine; (2) the presence of a component of thiamine uptake which is mediated by a hOCT-independent mechanism, probably corresponding to non-specific transport of thiamine.

In the present work, we also investigated regulation of thiamine transport in Caco-2 cells by phosphorylation/dephosphorylation processes. DB-AMPc, a PKA activator, vanadate, a protein tyrosine phosphatase (PTP) inhibitor, and levamisole, an alkaline phosphatase inhibitor, were tested. All of these substances significantly inhibited thiamine transport. These data confirm that phosphorylation/dephosphorylation mechanisms play an important role in regulation on apical uptake of thiamine in Caco-2 cells, as Said et al. (1999) already verified that thiamine uptake by Caco-2 was dependent on Ca^{2+}/calmodulin. In the present work, we further extend this conclusion by showing that it is also dependent on PKA, protein tyrosine phosphatase and alkaline phosphatase. Comparison of our results with previous results on ^{3}H-T^+ uptake in Caco-2 cells (Said et al., 1999) and ^{3}H-MPP^+ uptake in Caco-2 cells (OCT1- and OCT3-mediated; (Martel et al., 2002)) gives some interesting

results. Neither [^{3}H]-T$^+$ nor [^{3}H]-MPP$^+$ uptake were affected by a protein tyrosine kinase inhibitor, although a PTP inhibitor reduced [^{3}H]-T$^+$ uptake in the present work (Martel et al., 2002; Said et al., 1999), and [^{3}H]-MPP$^+$ uptake was reduced by a PKC inhibitor (Martel et al., 2002). Overall, these data do not allow us to exclude the involvement of either the thiamine specific transporters (ThTr1 and /or ThTr2) or OCTs in [^{3}H]-T$^+$ uptake by Caco-2 cells (Hayeshi et al., 2008; Seithel et al., 2006). Affinity of these transporters for thiamine is probably different having ThTr1 and 2 higher affinities for thiamine than OCTs. However, the high concentrations of [^{3}H]-T$^+$ used in this study (400 nM) provided the conditions to prove the involvement of OCTs.

Finally, it was also observed that progesterone inhibited thiamine uptake. Interestingly enough, progesterone is an inhibitor of OCT1, OCT2 and OCT3 (Koepsell et al., 2007), which is in agreement with the hypothesis of an OCT involvement in intestinal thiamine uptake. P-glycoprotein (P-gp) is an extrusion transporter on the apical membrane of enterocytes. Because progesterone is also a P-gp inhibitor, the involvement of this transporter on thiamine handling by the cells could be also suggested. However, the effect of progesterone does not seem to be over P-gp, because inhibition of P-gp would be expected to cause an increase in the accumulation of thiamine in the cells, which was not the case.

5. Conclusion

Our results are compatible with the possibility of thiamine being transported not only by ThTr1 and/or ThTr2, but also by members of the OCT family of transporters (most probably OCT1 and/or OCT3), thus sharing the same transporters with several other organic cations at the small intestinal level. The hypothesis of thiamine being transported by OCT family members is supported by the following data: i) pH-dependence characteristics of [^{3}H]-T$^+$ uptake by Caco-2 cells are consistent with a T$^+$/H$^+$ cotransport, which is not the proposed mechanism of either ThTr1- and ThTr1-mediated transport; ii) some organic cations known as OCT substrates/inhibitors (MPP$^+$, decynium22, clonidine, dopamine, serotonin) inhibited [^{3}H]-T$^+$ uptake; iii) the OCT inhibitor progesterone inhibited [^{3}H]-T$^+$ uptake. Moreover, the existence of OCT-mediated thiamine uptake in Caco-2 cells would also explain the previous two observations: i) a significant reduction in organic cation uptake in Caco-2 cells produced by thiamine (Calhau et al., 2003); and (ii) cells transfected with EMT were able to inhibit thiamine transport (Dutta et al., 1999). Finally, the involvement also of OCT transporters in the intestinal absorption of thiamine, apart from ThTr1 and ThTr2, would explain the inconsistency between pharmacological studies that reveal a great capacity of the intestine to transport thiamine and molecular biology studies in which the presence of ThTr1 in the intestinal epithelia would not be significant and would be insufficient to explain this tissue ability to transport this vitamin (Rindi and Laforenza, 2000).

Acknowledgements

This work was supported by FCT, COMPETE, QREN and FEDER (PTDC/SAU-OSM/102239/2008 and PTDC/QUI/65501/2006), two postdoctoral research fellows (SFRH/BPD/75294/2010, SFRH/BPD/40110/2007) and a PhD fellow (SFRH/BD/78367/2011).

Clara Lemos has an Humboldt Research Fellowship for Postdoctoral Researchers from the Alexander von Humboldt Foundation.

References

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Busch, A.E., Karbach, U., Miska, D., Gorboulev, V., Akhoundova, A., Volk, C., Arndt, P., Ulzheimer, J.C., Sonders, M.S., Baumann, C., Waldegger, S., Lang, F., Koepsell, H., 1998. Human neurons express the polyspecific cation transporter hOCT2, which translocates monoamine neurotransmitters, amantadine, and memantine. Mol. Pharmacol. 54, 342–352.

Calhau, C., Martel, F., Hipolito-Reis, C., Azevedo, I., 2003. Effect of thiamine on 3H-MPP+ uptake by Caco-2 cells. Pharmacol. Res. 48, 579–584.

Delie, F., Rubas, W., 1997. A human colonic cell line sharing similarities with enterocytes as a model to examine oral absorption: advantages and limitations of the Caco-2 model. Crit. Rev. Ther. Drug Carrier Syst. 14, 221–286.

Diaz, G.A., Banikazemi, M., Oishi, K., Desnick, R.J., Gelb, B.D., 1999. Mutations in a new gene encoding a thiamine transporter cause thiamine-responsive megaloblastic anaemia syndrome. Nat. Genet. 22, 309–312.

Dudeja, P.K., Tyagi, S., Kavilaveettil, R.J., Gill, R., Said, H.M., 2001. Mechanism of thiamine uptake by human jejunal brush-border membrane vesicles. Am. J. Physiol. Cell Physiol. 281, C786–C792.

Dudeja, P.K., Tyagi, S., Gill, R., Said, H.M., 2003. Evidence for a carrier-mediated mechanism for thiamine transport to human jejunal basolateral membrane vesicles. Dig. Dis. Sci. 48, 109–115.

Dutta, B., Huang, W., Molero, M., Kekuda, R., Leibach, F.H., Devoe, L.D., Ganapathy, V., Prasad, P.D., 1999. Cloning of the human thiamine transporter, a member of the folate transporter family. J. Biol. Chem. 274, 31925–31929.

Fabian, E., Elmadfa, I., 2008. Nutritional situation of the elderly in the European Union: data of the European Nutrition and Health Report (2004). Ann. Nutr. Metab. 52 (Suppl 1), 57–61.

Frank, T., Czeche, K., Bitsch, R., Stein, G., 2000. Assessment of thiamin status in chronic renal failure patients, transplant recipients and hemodialysis patients receiving a multivitamin supplementation. Int. J. Vitam. Nutr. Res. 70, 159–166.

Ganapathy, V., Smith, S.B., Prasad, P.D., 2004. SLC19: the folate/thiamine transporter family. Pflugers Arch. 447, 641–646.

Grundemann, D., Liebich, G., Kiefer, N., Koster, S., Schomig, E., 1999. Selective substrates for non-neuronal monoamine transporters. Mol. Pharmacol. 56, 1–10.

Hayeshi, R., Hilgendorf, C., Artursson, P., Augustijns, P., Brodin, B., Dehertogh, P., Fisher, K., Fossati, L., Hovenkamp, E., Korjamo, T., Masungi, C., Maubon, N., Mols, R., Mullertz, A., Monkkonen, J., O'Driscoll, C., Oppers-Tiemissen, H.M., Ragnarsson, E.G., Rooseboom, M., Ungell, A.L., 2008. Comparison of drug transporter gene expression and functionality in Caco-2 cells from 10 different laboratories. Eur. J. Pharm. Sci. 35, 383–396.

Keating, E., Lemos, C., Monteiro, R., Azevedo, I., Martel, F., 2004. The effect of a series of organic cations upon the plasmalemmal serotonin transporter, SERT. Life Sci. 76, 103–119.

Keating, E., Lemos, C., Azevedo, I., Martel, F., 2006. Characteristics of thiamine uptake by the BeWo human trophoblast cell line. J. Biochem. Mol. Biol. 39, 383–393.

Koepsell, H., Lips, K., Volk, C., 2007. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. Pharm. Res. 24, 1227–1251.

Koike, H., Sobue, G., 2006. Alcoholic neuropathy. Curr. Opin. Neurol. 19, 481–486.

Kumar, N., 2010. Neurologic presentations of nutritional deficiencies. Neurol. Clin. 28, 107–170.

Labay, V., Raz, T., Baron, D., Mandel, H., Williams, H., Barrett, T., Szargel, R., McDonald, L., Shalata, A., Nosaka, K., Gregory, S., Cohen, N., 1999. Mutations in SLC19A2 cause thiamine-responsive megaloblastic anaemia associated with diabetes mellitus and deafness. Nat. Genet. 22, 300–304.

Laforenza, U., Orsenigo, M.N., Rindi, G., 1998. A thiamine/H+ antiport mechanism for thiamine entry into brush border membrane vesicles from rat small intestine. J. Membr. Biol. 161, 151–161.

Lengyel, C.O., Whiting, S.J., Zello, G.A., 2008. Nutrient inadequacies among elderly residents of long-term care facilities. Can. J. Diet. Pract. Res. 69, 82–88.

Martel, F., Calhau, C., Azevedo, I., 2000. Characterization of the transport of the organic cation [3H]MPP+ in human intestinal epithelial (Caco-2) cells. Naunyn Schmiedebergs Arch. Pharmacol. 361, 505–513.

Martel, F., Grundemann, D., Calhau, C., Schomig, E., 2001. Apical uptake of organic cations by human intestinal Caco-2 cells: putative involvement of ASF transporters. Naunyn Schmiedebergs Arch. Pharmacol. 363, 40–49.

Martel, F., Keating, E., Calhau, C., Azevedo, I., 2002. Uptake of (3)H-1-methyl-4-phenylpyridinium ((3)H-MPP(+)) by human intestinal Caco-2 cells is regulated by phosphorylation/dephosphorylation mechanisms. Biochem. Pharmacol. 63, 1565–1573.

Morley, J.E., 2010. Nutrition and the brain. Clin. Geriatr. Med. 26, 89–98.

Olsen, A., Halkjaer, J., van Gils, C.H., Buijsse, B., Verhagen, H., Jenab, M., Boutron-Ruault, M.C., Ericson, U., Ocke, M.C., Peeters, P.H., Touvier, M., Niravong, M., Waaseth, M., Skeie, G., Khaw, K.T., Travis, R., Ferrari, P., Sanchez, M.J., Agudo, A., Overvad, K., Linseisen, J., Weikert, C., Sacerdote, C., Evangelista, A., Zylis, D., Tsiotas, K., Manjer, J., van Guelpen, B., Riboli, E., Slimani, N., Bingham, S., 2009. Dietary intake of the water-soluble vitamins B1, B2, B6, B12 and C in 10 countries in the European Prospective Investigation into Cancer and Nutrition. Eur. J. Clin. Nutr. 63 (Suppl 4), S122–S149.

Rajgopal, A., Edmondnson, A., Goldman, I.D., Zhao, R., 2001. SLC19A3 encodes a second thiamine transporter ThTr2. Biochim. Biophys. Acta 1537, 175–178.

Rindi, G., Laforenza, U., 2000. Thiamine intestinal transport and related issues: recent aspects. Proc. Soc. Exp. Biol. Med. 224, 246–255.

Said, H.M., Ortiz, A., Kumar, C.K., Chatterjee, N., Dudeja, P.K., Rubin, S., 1999. Transport of thiamine in human intestine: mechanism and regulation in intestinal epithelial cell model Caco-2. Am. J. Physiol. 277, C645–C651.

Said, H.M., Balamurugan, K., Subramanian, V.S., Marchant, J.S., 2004. Expression and functional contribution of hTHTR-2 in thiamin absorption in human intestine. Am. J. Physiol. Gastrointest. Liver Physiol. 286, G491–G498.

Saito, N., Kimura, M., Kuchiba, A., Itokawa, Y., 1987. Blood thiamine levels in outpatients with diabetes mellitus. J. Nutr. Sci. Vitaminol. (Tokyo) 33, 421–430.

Seithel, A., Karlsson, J., Hilgendorf, C., Bjorquist, A., Ungell, A.L., 2006. Variability in mRNA expression of ABC- and SLC-transporters in human intestinal cells: comparison between human segments and Caco-2 cells. Eur. J. Pharm. Sci. 28, 291–299.

Soukoulis, V., Dihu, J.B., Sole, M., Anker, S.D., Cleland, J., Fonarow, G.C., Metra, M., Pasini, E., Strzelczyk, T., Taegtmeyer, H., Gheorghiade, M., 2009. Micronutrient deficiencies an unmet need in heart failure. J. Am. Coll. Cardiol. 54, 1660–1673.

Subramanya, S.B., Subramanian, V.S., Said, H.M., Chronic alcohol consumption and intestinal thiamin absorption: effects on physiological and molecular parameters of the uptake process. Am J Physiol Gastrointest Liver Physiol 299, G23-31.

Tallaksen, C.M., Bell, H., Bohmer, T., 1992. The concentration of thiamin and thiamin phosphate esters in patients with alcoholic liver cirrhosis. Alcohol Alcohol. 27, 523–530.

Thornalley, P.J., Babaei-Jadidi, R., Al Ali, H., Rabbani, N., Antonysunil, A., Larkin, J., Ahmed, A., Rayman, G., Bodmer, C.W., 2007. High prevalence of low plasma thiamine concentration in diabetes linked to a marker of vascular disease. Diabetologia 50, 2164–2170.

Wooley, J.A., 2008. Characteristics of thiamin and its relevance to the management of heart failure. Nutr. Clin. Pract. 23, 487–493.

Yee, S., 1997. In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man—fact or myth. Pharm. Res. 14, 763–766.