Transport of thiamine in human intestine: mechanism and regulation in intestinal epithelial cell model Caco-2

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Veterans Affairs Medical Center, Long Beach 90822; University of California Irvine, Irvine 92717; Wadsworth Veterans Affairs Medical Center and University of California Los Angeles, Los Angeles, California 90073; and Westside Veterans Affairs Medical Center and University of Illinois-Chicago, Chicago, Illinois 60612

Said, Hamid M., Alvaro Ortiz, Chandira K. Kumar, Nabendu Chatterjee, Pradeep K. Dudeja, and Stanley Rubin. Transport of thiamine in human intestine: mechanism and regulation in intestinal epithelial cell model Caco-2. Am. J. Physiol. 277 (Cell Physiol. 46): C645–C651, 1999.—The present study examined the intestinal uptake of thiamine (vitamin B1) using the human-derived intestinal epithelial cells Caco-2 as an in vitro model system. Thiamine uptake was found to be 1) temperature and energy dependent and occurred with minimal metabolic alteration; 2) pH sensitive; 3) Na+ independent; 4) saturable as a function of concentration with an apparent Michaelis-Menten constant of 3.18 ± 0.56 μM and maximal velocity of 13.37 ± 0.94 pmol·mg protein·1·min-1; 5) inhibited by the thiamine structural analogs amprolium and oxythiamine, but not by unrelated organic cations tetraethylammonium, N-methyl-Nicotinamide, and choline; and 6) inhibited in a competitive manner by amiloride with an inhibition constant of 0.2 mM. The role of specific protein kinase-mediated pathways in the regulation of thiamine uptake by Caco-2 cells was also examined using specific modulators of these pathways. The results showed possible involvement of a Ca2+/calmodulin (CaM)-mediated pathway in the regulation of thiamine uptake. No role for protein kinase C- and protein tyrosine kinase-mediated pathways in the regulation of thiamine uptake was evident. These results demonstrate the involvement of a carrier-mediated system for thiamine uptake by Caco-2 intestinal epithelial cells. This system is Na+ independent and is different from the transport systems of organic cations. Furthermore, a CaM-mediated pathway appears to play a role in regulating thiamine uptake in these cells.

Human intestinal cells; transport mechanism; transport regulation

Thiamine (vitamin B1) plays a critical role in normal carbohydrate metabolism and thus is essential for normal cellular functions and growth. Thiamine deficiency in humans leads to a variety of clinical abnormalities, including cardiovascular disorders (e.g., peripheral vasodilatation, biventricular myocardial failure, edema, and potentially acute fulminant cardiovascular collapse) and neurological disorders (e.g., confusion, disordered ocular motility, neuropathy, ataxia of gait) (2, 34, 38). Thiamine deficiency occurs in a high percentage of alcoholics (8, 19, 21, 33, 36, 37), where impairment in the intestinal absorption of the vitamin is believed to be a contributing factor (37). Deficiency of thiamine also occurs in patients with diabetes, coeliac disease (22), renal diseases (26), and in patients fed intravenously for long periods (22), as well as in the elderly (23). Furthermore, thiamine deficiency has also been reported in thiamine-responsive megaloblastic anemia (1, 28), where impairment in thiamine intestinal absorption and cellular uptake is believed to be a contributing factor (28).

Humans and other mammals cannot synthesize thiamine but instead must obtain the vitamin from exogenous sources via intestinal absorption. Thus the intestine plays a central role in maintaining normal thiamine body homeostasis. Dietary thiamine exists mainly in the phosphorylated forms (predominantly as thiamine pyrophosphate) that are hydrolyzed to free thiamine in the intestinal lumen before absorption (26). The mechanism of intestinal thiamine transport has been studied in a number of animal species (5, 9, 11, 13, 16), but less is known about the mechanism of thiamine transport in the human intestine. Furthermore, nothing is known about the cellular regulation of the intestinal thiamine uptake process. This is despite the fact that recent studies have shown that intestinal transport of a variety of substrates (including that of the water-soluble vitamins folate, biotin, and riboflavin) is regulated by specific intracellular regulatory mechanisms (4, 6, 7, 17, 30). Our aim in this study was, therefore, to study the mechanism and cellular regulation of thiamine uptake by the human intestine using the human-derived cultured intestinal epithelial cell line Caco-2 as a model. We chose these cells because previous studies have shown that postconfluent and differentiated Caco-2 cells possess many of the structural and functional characteristics of the native enterocyte, including similar transport mechanisms and regulatory pathways (20, 25). Our results showed that thiamine uptake by these cells occurs via a specialized, Na+-independent, carrier-mediated process that appears to be under the regulation of a Ca2+/-calmodulin (CaM)-mediated intracellular pathway.

MATERIALS AND METHODS

Custom-made [3H]thiamine (sp act 555 GBq/mmol; radiochemical purity >98%) was purchased from American Radio-labeled Chemicals, St Louis, MO. The radiochemical purity of [3H]thiamine was checked routinely before use by means of TLC with the use of cellulose-precocated thin-layer plates and a solvent system of isopropanol-acetate buffer (0.5 M, pH 2.5). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Caco-2 cells were grown as we described previously (30). Uptake studies were performed on confluent monolayers 3–5 days following confluence. Uptake of thiamine was examined in cells incubated in Kreb's-Ringer buffer (in mM: 133 NaCl, 4.93 KCl, 1.23 MgSO₄, 0.85 CaCl₂, 5 glucose, 5 glutamine, 10 HEPES, and 10 MES, pH 7.4, unless otherwise specified) at 37°C. Labeled and unlabeled thiamine were added to the incubation medium at the onset of the uptake experiment. In certain experiments, cells were pretreated with the compound under study for a specific period of time before the addition of thiamine and the start of uptake experiments. Uptake was examined over a period of 3 min (unless otherwise specified), and the reaction was terminated by the addition of 2 ml of ice-cold buffer followed by immediate aspiration. Cells were then rinsed twice with ice-cold buffer, digested with 1 ml of 1 N NaOH, neutralized with HCl, and then counted for radioactivity. The protein content of cell digest was measured in parallel wells using a Bio-Rad kit (Bio-Rad, Richmond, VA).

Data are means ± SE of multiple separate uptake determinations and were expressed in term of picomoles or femtomoles per milligram protein per unit time. Statistical differences were analyzed by ANOVA or Student's t-test, with statistical significance being set at 0.05 (P < 0.05). Quantitative variations in the absolute amounts of thiamine uptake were observed in certain experiments, especially those in which cells were preincubated for 1 h before initiation of thiamine uptake measurement. For this reason, appropriate controls were simultaneously performed with each set of experiments. Kinetic parameters of thiamine uptake [i.e., the apparent Michaelis-Menten constant (Kₘ) and maximal velocity (Vₘₚ)] were calculated using a computerized model of the Michaelis-Menten equation as described previously by Wilkinson (39).

RESULTS

Uptake of thiamine as a function of time, temperature, incubation buffer pH, and Na⁺. Figure 1 depicts the uptake of thiamine as a function of time at two concentrations: low (0.1 µM) and high (10 µM). Uptake was found to be linear with time for 10 min of incubation at both concentrations, and rates were 0.19 and 3.28 pmol·mg protein⁻¹·min⁻¹, respectively. A 3-min incubation time was used as the standard time in all subsequent studies.

We also examined the metabolic form of the transported radioactivity following 3- and 10-min incubations of cells with 0.04 µM [³H]thiamine. Cellulose precoated TLC plates and a solvent system of isopropanol-acetate buffer (0.5 M, pH 4.5)-water (65:15:20, vol/vol/vol) were used (see MATERIALS AND METHODS). The results showed that the majority (96% in both 3- and 10-min incubations) of the radioactivity taken up by the cells was in the form of intact thiamine.

In a separate experiment, the effect of incubation temperature (37, 22, and 4°C) on thiamine (0.02 µM) uptake was examined. Uptake was found to decrease progressively with decreasing incubation temperature (223.7 ± 3.6, 134.82 ± 8.2, and 38.4 ± 4.3 fmol·mg protein⁻¹·3 min⁻¹ at 37, 22, and 4°C, respectively).

The effect of varying the incubation buffer pH over the range of 5–8 on thiamine (0.02 µM) uptake was also tested. The results showed a progressive decrease in thiamine uptake as a function of decreasing the incubation buffer pH from 8 to 5 (Fig. 2). We also investigated the role of Na⁺ in the incubation medium on thiamine uptake by Caco-2 cells. This was performed by examining the effect of replacing Na⁺ in the incubation medium with an equimolar concentration of NH₄⁺ or mannitol. The results showed a slight but statistically insignificant decrease in thiamine uptake on Na⁺ removal [198.6 ± 5.3, 175.1 ± 19.2, and 174.7 ± 12.4 fmol·mg protein⁻¹·3 min⁻¹ in the presence of Na⁺ (control) as well as in the absence of Na⁺ and the presence of NH₄⁺ and mannitol, respectively]. The role of Na⁺ in thiamine uptake was also investigated by examining the effect of pretreating the cells (for 1 h) with the Na⁺-K⁺-ATPase inhibitor ouabain (1 mM) on the uptake of the substrate. The result showed no inhibition of thiamine (0.02 µM) uptake by this compound (114.6 ± 3.2 and 112 ± 1.2 fmol·mg protein⁻¹·3 min⁻¹ for control and ouabain-pretreated cells, respectively).
Uptake of thiamine as a function of concentration. Figure 3 depicts the results on the initial rate of thiamine uptake as a function of increasing the vitamin concentration in the incubation medium (0.02–10 µM). Uptake of thiamine was found to include a saturable component. Uptake by this component was calculated by subtracting uptake by simple diffusion from total uptake at each thiamine concentration. Uptake by simple diffusion was calculated from the slope of the uptake line between uptake at high thiamine concentration (1 mM) and the point of origin. Apparent \( K_m \) and \( V_{max} \) of the saturable component were then calculated as described in MATERIALS AND METHODS and found to be 3.18 ± 0.56 µM and 13.37 ± 0.94 pmol·mg protein\(^{-1}\)·min\(^{-1}\), respectively.

Effect of thiamine structural analogs and unrelated organic cations on the uptake of \(^{3}H \)thiamine. In these studies, we examined the effect of different concentrations of the thiamine structural analogs amprolium and oxythiamine on the uptake of \(^{3}H \)thiamine. The results showed that both compounds caused a concentration-dependent inhibition of \(^{3}H \)thiamine uptake; the inhibition was found (by the Dixon method) to be competitive in nature with calculated inhibition constants (\( K_i \)) of 7.8 and 28.7 µM for amprolium and oxythiamine, respectively (Fig. 4).

In other studies, we examined the effect of different concentrations of the organic cations tetrathylammonium (TEA), N-methylnicotinamide (NMN), and choline on \(^{3}H \)thiamine (0.02 µM) uptake. The effect of unlabeled thiamine (25 µM) in these studies served as a positive control. With the exception of unlabeled thiamine, none of the other compounds examined significantly affected \(^{3}H \)thiamine uptake (Table 1).

In another study, we examined the effect of various transport inhibitors DIDS, probenecid, furosemide, and amiloride (all at 1 mM concentration) on the uptake of 0.02 µM thiamine by Caco-2 cells. The results showed that, although DIDS, probenecid, and furosemide do not have an effect on thiamine uptake, amiloride was found to cause a marked inhibition of the vitamin uptake (238 ± 6.3, 223 ± 12.5, 220.3 ± 6.7, and 72.4 ± 5.8 fmol·mg protein\(^{-1}\)·min\(^{-1}\) for control and in the presence of DIDS, probenecid, furosemide, and amiloride, respectively). Amiloride inhibition was found (by the Dixon method) to be competitive in nature with a \( K_i \) value of 0.27 mM (Fig. 5).

Regulation of thiamine uptake: role of intracellular protein kinase-mediated pathways. In these studies we examined the possible regulation of the thiamine uptake process by specific intracellular protein kinase-mediated pathways. Specifically, we investigated the possible role of CaM-, protein kinase C (PKC)-, and protein tyrosine kinase (PTK)-mediated pathways in the regulation of thiamine uptake by examining the effect of pretreating Caco-2 cells (for 1 h) with specific modulators of these pathways on the vitamin uptake process.

Pretreatment of cells with the inhibitors of the CaM-mediated pathway trifluoroperazine (TFP), calmidazolium, and 1-[N-0-bis(5-isquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) led to a significant inhibition of thiamine (0.02 µM) uptake (Table 2). To further characterize the effect of these inhibitors on thiamine uptake, we examined the effect
of a representative compound, TFP, on kinetic parameters of the carrier-mediated thiamine uptake process. The results (Fig. 6) showed that TFP (75 µM) caused a significant (P < 0.01) inhibition of Vmax but not of the apparent Km of the thiamine uptake process (Vmax = 12.4 ± 0.78 and 8.32 ± 0.35 fmol·mg protein⁻¹·3 min⁻¹ and apparent Km = 2.6 ± 0.44 and 3.15 ± 0.34 µM for control and TFP-pretreated cells, respectively).

The possible role of a PKC-mediated pathway in the regulation of thiamine uptake by Caco-2 cells was investigated by examining the effect of pretreatment of the cells with modulators of PKC activity [phorbol 12-myristate 13-acetate (PMA), staurosporine, and bis-indolylmaleimide (Bis I)] on thiamine (0.02 µM) uptake. The results showed that none of the PKC modulators had an effect on thiamine uptake (119 ± 5.4, 114.9 ± 3.1, 129.9 ± 7.1, and 112.6 ± 5.2 fmol·mg protein⁻¹·3 min⁻¹ for control and in cells pretreated with 10 µM PMA, 10 µM staurosporine, and 1 µM Bis I, respectively). We also examined the effect of pretreating Caco-2 cells with inhibitors of PTK activity on thiamine (0.02 µM) uptake. The results showed that, although the PTK inhibitors genistein (100 µM) and tyrphostin A25 (10 µM) inhibit thiamine uptake, equimolar concentrations of their negative controls genistin and tyrphostin A1, respectively, also cause the same degree of inhibition (112 ± 6.6, 89.4 ± 6.1, 87.1 ± 2.9, 78.2 ± 13.7, and 74.3 ± 9.7 fmol·mg protein⁻¹·3 min⁻¹ for control and in cells pretreated with genistin, tyrphostin A25, genistin, and tyrphostin A1, respectively). These findings indicate that the inhibition caused by genistin and tyrphostin A25 is nonspecific in nature.

DISCUSSION

In this study, we examined the mechanism and cellular regulation of the thiamine uptake process by the human intestine using the human-derived intestinal epithelial cell line Caco-2 as an in vitro model system. The results showed that thiamine uptake by Caco-2 cells is linear for up to 10 min incubation and occurred with minimal metabolic alteration in the transported substrate. Uptake was also found to be temperature and energy dependent, as indicated by the significant inhibition of thiamine uptake caused by decreasing incubation temperature and by metabolic

Table 1. Effect of unrelated organic cations and that of unlabeled thiamine on the uptake of [³H]thiamine by confluent monolayers of Caco-2 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration, µM</th>
<th>Uptake, fmol·mg protein⁻¹·3 min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>239.3 ± 28.9*</td>
</tr>
<tr>
<td>TEA</td>
<td>25</td>
<td>236.5 ± 33.5*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>214.4 ± 21.6*</td>
</tr>
<tr>
<td>NMN</td>
<td>25</td>
<td>226.0 ± 26.6*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>236.0 ± 30.8*</td>
</tr>
<tr>
<td>Choline</td>
<td>25</td>
<td>231.7 ± 23.2*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>226.8 ± 29.5*</td>
</tr>
<tr>
<td>Thiamine</td>
<td>25</td>
<td>45.9 ± 4.2†</td>
</tr>
</tbody>
</table>

Values are means ± SE of 8–9 separate uptake determinations performed on three separate occasions. Cells were incubated (37°C) in Krebs-Ringer buffer (pH 7.4). [³H]thiamine (0.02 µM) was added at the onset of 3-min incubation. TEA, tetraethylammonium; NMN, N-methylnicotinamide; NS, not significant. *P = NS, †P < 0.01, calculated using ANOVA; comparison was made relative to simultaneously performed control.

Fig. 4. Dixon plot of the effect of amprolium (A) and oxythiamine (B) on thiamine uptake by confluent monolayers of Caco-2 cells. Cells were incubated at 37°C in Krebs-Ringer buffer (pH 7.4) in the presence of 0.1 µM (○) and 4.0 µM (●) [³H]thiamine and different concentrations of the structural analog under study. Thiamine uptake was measured during initial rate of uptake (3 min), and results were applied to Dixon plot as shown. Results are means ± SE of 3–7 separate uptake determinations performed on 2 separate occasions.

Fig. 5. Dixon plot of the effect of amiloride on thiamine uptake by confluent monolayers of Caco-2 cells; same as Fig. 4, except that different concentrations of amiloride were used.
Table 2. Effect of inhibitors of CaM-mediated pathway on thiamine uptake by confluent monolayers of Caco-2 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration, µM</th>
<th>Uptake, fmol·mg protein⁻¹·3 min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>90.0 ± 2.2</td>
</tr>
<tr>
<td>TFP</td>
<td>50</td>
<td>73.6 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>48.1 ± 4.7</td>
</tr>
<tr>
<td>Calmidazolium</td>
<td>10</td>
<td>65.4 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>56.6 ± 1.9</td>
</tr>
<tr>
<td>KN-62</td>
<td>25</td>
<td>56.3 ± 3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are means ± SE of 6–7 separate uptake determinations performed on 2 separate occasions. Cells were preincubated for 1 h with the indicated compound in Krebs-Ringer buffer (pH 7.4). [³H]thiamine (0.02 µM) was then added and incubation proceeded for 3 min (i.e., initial rate). TFP, trifluoroperazine. P < 0.01, calculated using ANOVA; comparison was made relative to simultaneously performed control.

Inhibitors, respectively. Decreasing incubation buffer pH from 8 to 5 was found to lead to a progressive inhibition of thiamine uptake. Although the exact cause of this inhibition is not clear, it cannot be attributed to changes in the ionic state of the thiamine molecule, since thiamine exists as a monovalent cation over the pH range examined (27). Further studies are required to clarify this issue. The uptake process of thiamine was found to be Na⁺ independent in nature, as indicated by the lack of significant inhibition of substrate uptake when Na⁺ was removed from the incubation medium, as well as by the lack of effect on thiamine uptake when the cells were pretreated with the Na⁺-K⁺-ATPase inhibitor ouabain. This conclusion is in agreement with recent findings in our laboratory using purified human jejunal brush-border membrane vesicles (unpublished observations) and with the findings of Laforenza et al. (18) using human intestinal biopsy specimens. It is, however, in contrast with the findings of Hoyumpa et al. (14) who reported that thiamine uptake by human intestinal biopsies is Na⁺ dependent.

Uptake of thiamine by Caco-2 cells was found to involve a carrier-mediated process. This is indicated both by the saturation of thiamine uptake as a function of concentration and by the inhibition of [³H]thiamine uptake caused by the thiamine structural analogs amprolium and oxythiamine. The apparent Kᵣ of the carrier system of 3.18 ± 0.56 µM suggests that this system is capable of efficiently absorbing dietary thiamine, which has been estimated to exist in the lumen of the human intestine at a concentration range of 0.1–2.0 µM (13). The observation that oxythiamine is a weaker inhibitor of the thiamine uptake process compared with amprolium suggests that the amino group at carbon-4 of the pyrimidine moiety of the thiamine molecule is important for the interaction of thiamine with its carrier protein, whereas the sulfur of the thiazol moiety (the sulfur exists in the molecule of oxythiamine but not in the molecule of amprolium) is not important. The identified carrier system for transport of the cationic thiamine appears to be different from the transport systems described for organic cations in human renal and liver cells (10, 40). This conclusion is based on the observation that high concentrations of the organic cations TEA, NMN, and choline (substrates for the organic cation transport systems) failed to inhibit the uptake of physiological concentrations of thiamine.

The uptake process of thiamine was found to be insensitive to the effect of the membrane transport inhibitors DIDS, probenecid, and furosemide. The finding of a lack of effect by furosemide on thiamine uptake suggests that the observed thiamine deficiency and suboptimal conditions observed in patients on long-term therapy with this diuretic agent (32) are not due to inhibition of thiamine transport at the intestinal level. Other mechanisms such as altered renal handling of thiamine could be involved, as has been previously suggested (32). In contrast to the lack of effect of these inhibitors on thiamine uptake, the diuretic amiloride, a classical inhibitor of Na⁺/H⁺ exchangers, was found to cause a concentration-dependent, competitive inhibition of thiamine uptake with a Kᵢ value of 0.27 mM. Such an interaction between thiamine and amiloride at the level of cell membrane transport has also been observed in neuroblastoma cells (3). Together, these findings highlight the need for further studies to assess the nutritional significance of such an interaction on the normal thiamine body homeostasis, especially in patients treated chronically with amiloride.

After the characterization of the mechanism of thiamine uptake by Caco-2 cells, we used these cells to study possible regulation of the vitamin uptake process by specific intracellular regulatory pathways. We focused on the possible role of protein kinase-mediated pathways, especially those that have been shown to play a significant regulatory role in the regulation of transport of other substrates in intestinal and other epithelia (4, 6, 7, 17, 30). The results showed that...
inhibition of the CaM-mediated pathway resulted in a significant inhibition of thiamine uptake by these cells. The effect of one such inhibitor (TFP) was further characterized by testing its effect on the kinetic parameters of the carrier-mediated thiamine uptake process. The results showed that treatment with TFP decreases V_{max} but not the apparent K_{m} of the thiamine uptake process. These findings suggest that the TFP effect is mediated via inhibition of the activity (and/or number) of the thiamine carriers at the luminal membrane, with no effect on carrier affinity. In contrast to the possible role of the CaM-mediated pathway, no role for PKC- and PTK-mediated pathways in the regulation of thiamine uptake was observed. This conclusion was based on the observation that pretreatment of cells with specific modulators of these pathways did not significantly affect thiamine uptake.

In summary, our findings demonstrate the involvement of a specialized, Na^{+}-independent, carrier-mediated system for thiamine uptake by Caco-2 cells. Furthermore, this system appears to be under the regulation of a CaM-mediated pathway.

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