Pyridoxine uptake by colonocytes: a specific and regulated carrier-mediated process

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Said ZM, Subramanian VS, Vaziri ND, Said HM. Pyridoxine uptake by colonocytes: a specific and regulated carrier-mediated process. Am J Physiol Cell Physiol 294: C1192–C1197, 2008. First published March 19, 2008; doi:10.1152/ajpcell.00015.2008.—The water-soluble vitamin B₆ (pyridoxine) is important for normal cellular functions, growth, and development. The vitamin is obtained from two exogenous sources: a dietary source, which is absorbed in the small intestine, and a bacterial source, where the vitamin is synthesized in significant quantities by the normal microflora of the large intestine. Evidence exists to suggest the bioavailability of the latter source of the vitamin, but nothing is known about the mechanism involved and its regulation. In this study, we addressed these issues using young adult mouse colonic epithelial (YAMC) cells and human colonic apical membrane vesicles (AMV) as models and using [³H]pyridoxine as the uptake substrate. The results showed the initial rate of [³H]pyridoxine uptake by YAMC cells to be 1) energy- and temperature- (but not Na-) dependent and to occur without metabolic alteration in the transported substrate; 2) saturable as a function of concentration with an apparent Km of 2.1 ± 0.5 μM and 53.4 ± 4.3 pmol·mg protein⁻¹·min⁻¹ respectively; 3) cis-inhibited by unlabeled pyridoxine and its structural analogs, but not by the unconjugated compounds theophylline, penicillamine, and isoniazid; 4) trans-stimulated by unlabeled pyridoxine; 5) amiloride sensitive; and 6) regulated by extracellular and intracellular factors. Uptake of pyridoxine by native human colonic AMV was also found to involve a carrier-mediated process. These studies demonstrate, for the first time, the functional existence of a specific and regulatable carrier-mediated process for pyridoxine uptake by mammalian colonocytes.

THE WATER-SOLUBLE VITAMIN B₆ represents a group of three related compounds (pyridoxine, pyridoxal, and pyridoxamine) and their phosphorylated forms. Of these compounds, pyridoxal 5’-phosphate represents the major biologically active form of vitamin B₆, while pyridoxine is the form that is commonly used in nutritional supplements and vitamin preparations. Furthermore, both pyridoxine and pyridoxamine are converted into pyridoxal phosphate in the body. Vitamin B₆ plays an essential role as a cofactor in a variety of metabolic reactions that include amino acid (homocysteine), carbohydrate, and neurotransmitter metabolism, as well as sphingolipid biosynthesis (13). Recent studies have described a role for vitamin B₆ in the protection against reactive oxygen free radicals by showing that pyridoxine and pyridoxal are as effective antioxidants as vitamin C and vitamin E (2, 6). Deficiency of vitamin B₆ in humans, which could lead to a variety of clinical abnormalities that include neurological disorders, occurs in conditions like chronic alcoholism and diabetes, in patients with celiac and renal diseases, and following long-term use of hydrazines (e.g., isoniazid) and penicillamine (see Ref. 13 for review). On the other hand, pyridoxine supplementation is effective in the treatment of pyridoxine-dependent seizures in neonates and infants, caused by an autosomal recessive inborn error of metabolism that is believed to be due to an abnormality in cellular pyridoxine uptake (7).

Vitamin B₆ is synthesized by plant cells and most unicellular microorganisms, but humans and other mammals cannot synthesize the vitamin and thus must obtain it from exogenous sources via intestinal absorption. Two sources of vitamin B₆ are available to the intestine: a dietary source and a bacterial source; the latter source is in reference to the vitamin B₆ that is produced by the normal microflora of the large intestine. The mechanism of absorption of dietary vitamin B₆ in the small intestine has been studied using a variety of small intestinal preparations (8, 14, 15, 20, 23), with recent findings showing the existence of an efficient and specific carrier-mediated mechanism (20, 23). Nothing, however, is known about the mechanism of uptake of the bacterially produced vitamin B₆ in the large intestine, where a significant amount of the vitamin is produced and exists in the free form in the surrounding medium (i.e., rather than being trapped within the bacterial cells) (16) and thus is available for absorption. Evidence suggests that this source of vitamin B₆ is indeed bioavailable to the host comes from studies showing that the amount of the vitamin that is excreted is significantly higher than the total amount consumed orally (11). Our aim in the present study was to investigate the mechanism involved in vitamin B₆ (pyridoxine) uptake by mammalian colonocytes using the young adult mouse colonic epithelial (YAMC) cells and purified human colonic apical membrane vesicles (AMV) as models. The results showed, for the first time, the existence of a specific carrier-mediated mechanism for pyridoxine in mammalian colonocytes, which appears to be under extracellular and intracellular regulation.

MATERIALS AND METHODS

Materials. The YAMC cells were provided as a gift from Dr. Robert Whitehead (Vanderbilt University Medical Center, Nashville, TN) (17, 25). [³H]pyridoxine (specific activity of 20 Ci/mmol; radiochemical purity >97%) was obtained from ARC (St. Louis, MO). Unlabeled pyridoxine and all other chemicals and reagents were purchased from commercial sources and were of analytical grade.

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Cell culture and uptake assays. YAMC, conditionally immortalized murine colonic epithelial cells, were maintained at 33°C in Dulbecco’s modified Eagle’s medium (DMEM) without added pyridoxine (Life Technologies). Media were supplemented with 10% fetal bovine serum (FBS), glutamine (0.29 g/l), sodium bicarbonate (3.7 g/l), penicillin (100,000 U/l), and streptomycin (10 mg/l). Purified human colonic membrane vesicles were obtained from Dr. Pradeep Dudeja (University of Illinois at Chicago, Chicago, IL) and were isolated from organ donors as described by us previously (5).

\[^{3}H\]pyridoxine uptake in colonic membrane vesicle was measured at room temperature for 1 min by rapid filtration method (9) as previously described (5). Routine \[^{3}H\]pyridoxine uptake assay was performed using confluent monolayer (3–4 days after confluence) of YAMC in a 12-well plate. Protein concentrations were estimated on parallel wells using a protein assay kit (Bio-Rad). To determine the degree of pyridoxine metabolism after uptake by YAMC cells, a thin-layer chromatography (TLC) procedure employing cellulose gel–precoated plates and a solvent system of isopropanol/0.5 M acetate buffer (pH 4.5)/water (65/15/20, vol/vol/vol) was used as described previously (26).

Statistical analysis. Data of all uptake experiments are the results of at least three independent determinations and are expressed as means ± SE (in pmol·mg protein\(^{-1}\)·unit time\(^{-1}\) or fmol·mg protein\(^{-1}\)·unit time\(^{-1}\)). Statistical analysis was performed using ANOVA or Students \(t\)-test, with a significant \(P\) value set at <0.05.

Kinetic parameters of the saturable component of pyridoxine uptake were determined by subtracting the diffusion component [calculated from the slope of the line between uptake at high concentration (1 mM) and the point of origin] from total uptake; data were then applied to a computerized model of the Michaelis-Menten equation as described previously (26).

RESULTS

Uptake of pyridoxine by YAMC cells: time course; effect of buffer pH, \(Na^{+}\), and temperature; and possible metabolism during transport. Pyridoxine uptake by YAMC cells as a function of incubation time was linear for up to 10 min of incubation (rate = 170.5 fmol·mg protein\(^{-1}\)·unit time\(^{-1}\) and 17.9 pmol·mg protein\(^{-1}\)·unit time\(^{-1}\) for 15 nM and 3 \(\mu\)M, respectively; \(r = 0.99\) for both; Fig. 1). A 3-min incubation time was chosen to represent the initial rate of uptake in all subsequent investigations. The effect of incubation buffer pH on pyridoxine uptake by YAMC cells was examined to determine the role of \(H^{+}\) pyridoxine uptake. Uptake of pyridoxine (15 nM) was relatively high over the pH range of 6.5 to 8.0 but decreased at lower pH (Fig. 2). For this reason, an incubation buffer pH of 7.4 was used in all reported studies.

The role of \(Na^{+}\) in pyridoxine uptake was investigated by determining the effect of iso-osmotic replacement of \(Na^{+}\) in the incubation buffer with K\(^{+}\), Li\(^{+}\), or mannitol. The results showed similar initial rate of pyridoxine (15 nM) uptake in the presence and absence of \(Na^{+}\) (850 ± 40, 860 ± 80, 880 ± 30, and 830 ± 50 fmol·mg protein\(^{-1}\)·3 min\(^{-1}\) in the presence of \(Na^{+}\), K\(^{+}\), Li\(^{+}\), and mannitol, respectively). In a related study, we pretreated YAMC monolayer (for 30 min) with the Na\(^{+}\), K\(^{+}\)-ATPase inhibitor ouabain (10 mM) and examined the effect of that pretreatment on pyridoxine uptake. No significant inhibition was observed in the initial rate of pyridoxine (15 nM) uptake following pretreatment with ouabain (771 ± 38 and 845 ± 18 fmol·mg protein\(^{-1}\)·3 min\(^{-1}\) for control and ouabain-pretreated cells, respectively).

The effect of incubation temperature on \[^{3}H\]pyridoxine uptake by YAMC cells was also examined. Uptake was found to be highly temperature dependent, with a higher uptake at 37°C compared with 22°C (760 ± 50 and 220 ± 10 fmol·mg protein\(^{-1}\)·3 min\(^{-1}\), respectively).

The metabolic form of the transported radioactivity taken by YAMC cells following 10-min incubation with \[^{3}H\]pyridoxine (75 nM) was examined by means of TLC (see MATERIALS AND METHODS), with the majority (96%) of the transported substrate found to be in the form of intact pyridoxine.

Evidence for existence of a carrier-mediated process for pyridoxine uptake by colonocytes. The initial rate of pyridoxine uptake as a function of substrate concentration (0.1–10 \(\mu\)M) was examined, with the results showing evidence for the existence of a saturable uptake process (Fig. 3). Kinetic parameters of the saturable components were determined as described in MATERIALS AND METHODS and were found to be 2.1 ± 0.5 \(\mu\)M and 53.4 ± 4.3 pmol·mg protein\(^{-1}\)·3 min\(^{-1}\) for the apparent \(K_m\) and \(V_{max}\), respectively. This finding suggests involvement of a carrier-mediated process for pyridoxine uptake by YAMC cells. To further confirm the existence of a

Fig. 1. Uptake of pyridoxine by young adult mouse colonic epithelial (YAMC) cells as a function of incubation time. Confluent monolayer of YAMC cells was incubated at 37°C in Krebs-Ringer buffer (pH 7.4) for different time periods in the presence of 15 nM (A) or 3 \(\mu\)M (B) \[^{3}H\]pyridoxine. Data are means ± SE of 3–6 separate uptake determinations. When not shown, error bars are smaller than symbols.
carrier-mediated system for pyridoxine uptake and to develop an understanding of its specificity, we examined the effect of unlabeled pyridoxine and that of its related compounds pyridoxal, pyridoxal 5-phosphate, and pyridoxamine, as well as that of the unrelated isoniazid, penicillamine, theophylline, and homocystine (all at 50 μM) on the initial rate of [3H]pyridoxine (15 nM) uptake. The results showed that while unlabeled pyridoxine, pyridoxal, pyridoxal 5-phosphate, and pyridoxamine all cause significant (P < 0.01) inhibition in [3H]pyridoxine uptake, no such effect was seen with isoniazid, penicillamine, theophylline, and homocystine (Fig. 4).

In another study, we investigated possible trans-stimulation in [3H]pyridoxine transport across the YAMC cell membrane by unlabeled pyridoxine. In this experiment, we first preloaded the cells with 15 nM [3H]pyridoxine (for 10 min at 37°C) and then incubated the cells (for 10 min) in the absence and presence of 1 mM of unlabeled pyridoxine in the incubation buffer. The results showed the cellular content of [3H]radioactivity to be significantly (P < 0.05) lower in YAMC cells incubated in the presence of unlabeled pyridoxine in the incubation buffer compared with those incubated in its absence (cell content of [3H]radioactivity was 568 ± 2 and 805 ± 64 fmol·mg protein⁻¹·10 min⁻¹, respectively), indicating the ability of extracellular unlabeled pyridoxine to drive the efflux of [3H]pyridoxine from the cells.

We also aimed at establishing the existence of a carrier-mediated process for pyridoxine uptake in native human colonocytes. To this end, we used purified native human proximal colonic AMV isolated by an established procedure from the colonic mucosa of human organ donors (5) and examined the effect of unlabeled pyridoxine (1 mM) on the initial rate of [3H]pyridoxine (250 nM) uptake. Significant (P < 0.01) inhibition in [3H]pyridoxine uptake by unlabeled pyridoxine was observed (Fig. 5).

Effect of metabolic and transport inhibitors on [3H]pyridoxine uptake by YAMC cells. Energy dependence of the pyridoxine uptake process by YAMC cells was examined by testing the effect of pretreating the cells with the metabolic inhibitors 2,4-dinitrophenol (DNP; 1 mM) and iodoacetate (1 mM) for 30 min on the initial rate of pyridoxine (15 nM) uptake. The
results showed significant ($P < 0.01$ for both) inhibition in the vitamin uptake by both inhibitors (502 ± 11, 230 ± 20, and 237 ± 35 fmol·mg protein$^{-1}$·3 min$^{-1}$ for control and in the presence of DNP and iodoacetate, respectively).

The membrane transport inhibitor amiloride has been shown to inhibit pyridoxine uptake in mammalian and other cellular systems (3, 4, 22–24). We therefore examined its effect and that of other membrane transport inhibitors (DIDS, probenecid, and cyclohexamide) on the initial rate of $[^3]$H]pyridoxine (15 nM) uptake by YAMC cells. The results showed amiloride to cause a significant ($P < 0.01$) and concentration-dependent inhibition in pyridoxine uptake (737 ± 49, 297 ± 60, and 227 ± 56 fmol·mg protein$^{-1}$·3 min$^{-1}$ for control and in the presence of 0.1 and 1 mM amiloride, respectively). On the other hand, neither DIDS nor probenecid was found to affect the initial rate of pyridoxine uptake (737 ± 49, 720 ± 60, and 720 ± 50 fmol·mg protein$^{-1}$·3 min$^{-1}$ for control and in the presence of DIDS and probenecid, respectively).

Possible regulation of the pyridoxine uptake process in these investigations, we examined whether the pyridoxine uptake process of the YAMC cells is regulated by extracellular and intracellular factors. The extracellular factor that was selected for testing was increased extracellular pyridoxine level; YAMC cells were maintained (for 48 h) in a growth medium in the absence and presence of oversupplemented level (100 μM) of unlabeled pyridoxine. The results showed $[^3]$H]pyridoxine (15 nM) uptake by cells maintained in the presence of high pyridoxine level to be significantly ($P < 0.01$) lower than that of cells maintained in its absence (310 ± 30 and 930 ± 50 fmol·mg protein$^{-1}$·3 min$^{-1}$, respectively). This effect was specific for pyridoxine because uptake of the unrelated folic acid (8.6 nM; pH 5.5) was similar under the two pyridoxine-level conditions (1,840 ± 30 and 1,880 ± 70 fmol·mg protein$^{-1}$·3 min$^{-1}$ for cell growth in the absence and presence of pyridoxine oversupplementation, respectively). To determine the level at which this adaptive regulation in the pyridoxine uptake process is taking place, we first maintained YAMC cells (for 24 h) in pyridoxine-oversupplemented (100 μM) medium, then moved them to a medium that lacks pyridoxine oversupplementation in the absence or presence of the transcription inhibitor actinomycin D (0.23 μM) or DIDS and probenecid (40 μM). Incubation was then continued for an additional 24 h, followed by examination of the initial rate of $[^3]$H]pyridoxine (15 nM) uptake. As before, the uptake of cells maintained in the absence of pyridoxine oversupplementation was significantly ($P < 0.01$) higher than the uptake of cells maintained in the presence of pyridoxine oversupplementation. This induction in pyridoxine uptake was significantly ($P < 0.01$ for both) inhibited in the presence of actinomycin D and cyclohexamide in the growth medium (Fig. 6).

Possible regulation of the pyridoxine uptake process of YAMC cells by intracellular regulatory pathways focused on testing the effect of modulators of specific protein kinase-mediated pathways that include Ca$^{2+}$/CaM-, protein kinase A (PKA)-, protein kinase C (PKC)-, and nitric oxide (NO)-mediated pathways. The role for the Ca$^{2+}$/CaM-mediated pathway was tested by examining the effect of pretreating the cells (for 1 h) with specific inhibitors of this pathway (trifluoperazine (TFP), calmidazolium, and 1-[N-$O$-bis(5-isoquinolinesulfonyl)-N-methyl-$l$-tyrosyl]-4-phenyl piperezine (KN-62) (all at 50 μM)) on the initial rate of pyridoxine (15 nM) uptake. The results showed that these compounds cause significant ($P < 0.01$ for all) inhibition in pyridoxine uptake (620 ± 20, 350 ± 3, 190 ± 10, and 370 ± 40 fmol·mg protein$^{-1}$·3 min$^{-1}$ for control, following pretreatment with TFP, calmidazolium, and KN-62, respectively). On the other hand, pretreatment with modulators of the PKA-mediated pathway (cAMP and forskolin, both at 1 nM) did not affect pyridoxine uptake (540 ± 30, 610 ± 50, and 610 ± 20 fmol·mg protein$^{-1}$·3 min$^{-1}$ for control and following pretreatment with KN-62, respectively). Similarly, modulators of the PKC-mediated pathway (all at 1 μM) failed to affect pyridoxine uptake (560 ± 30, 520 ± 30, 510 ± 20, and 540 ± 20 fmol·mg protein$^{-1}$·3 min$^{-1}$ for control and following pretreatment with phorbol 12-myristate 13-acetate, chelistrymy, and staurosporine, respectively). Finally, we tested the potential role of the NO-mediated pathway in the regulation of pyridoxine uptake by YAMC cell by examining the effect of pretreating (1 h) the cells with the NO pathway modulators 8-bromo cGMP and S-nitroso-$N$-acetylpenicillamine (SNAP) (both at 1 mM), but neither compound led to a significant effect an pyridoxine uptake (615 ± 15, 780 ± 30, and 705 ± 60 fmol·mg protein$^{-1}$·3 min$^{-1}$ for control, 8-bromo cGMP, and SNAP, respectively).

**DISCUSSION**

As mentioned above, a considerable amount of free vitamin B$_6$ is synthesized by the normal microflora of the mammalian large intestine, and this vitamin exists in the colonic lumen and thus is available for absorption. Also, evidence exists to indicate that the large intestine is capable of absorbing this source of the vitamin, yet nothing is known about the mechanism involved in this absorption and its regulation. The aim of the present investigations was to address these issues using the cultured mouse colonic YAMC cells and native human colonic AMV as models, and pyridoxine as the substrate. The results
showed pyridoxine uptake to be pH sensitive, with an increase in uptake at 6.5 and higher pH but decreased uptake at lower incubation buffer pH. This characteristic of pyridoxine uptake by colonic YAMC cells is similar to its characteristics in renal epithelial cells, where again uptake was found to be higher at neutral/alkaline pH compared with acidic incubation buffer pH (22). Uptake of pyridoxine by YAMC cells was Na⁺ independent as iso-osmotic replacement of Na⁺ in the incubation buffer with other monovalent cations or mannitol, and pretreatment of the cells with the Na⁺/K⁺-ATPase inhibitor ouabain failed to affect the initial rate of the vitamin uptake. The latter finding on the lack of a role of Na⁺ in pyridoxine uptake is similar to what we have observed with a model of small intestinal epithelial cells (23).

The initial rate of pyridoxine uptake by YAMC cells was saturable as a function of increasing the substrate concentration in the incubation medium (apparent \( K_m = 2.1 \pm 0.5 \mu M \)), suggesting involvement of a carrier-mediated mechanism in the uptake process. This suggestion was confirmed by the finding of a significant cis-inhibition in the initial rate of \([3H]\)pyridoxine uptake by unlabeled pyridoxine and the pyridoxine-related compounds pyridoxal, pyridoxal 5-phosphate, and pyridoxine. Furthermore, the ability of unlabeled pyridoxine to trans-stimulate \([3H]\)pyridoxine efflux from preloaded YAMC cells lent further support for the involvement of a carrier-mediated mechanism. The inability of the unrelated penicillamine, theophylline, and homocystine to affect the initial rate of \([3H]\)pyridoxine uptake by YAMC cells demonstrates specificity of the colonic pyridoxine uptake process. In addition, since penicillamine, theophylline, and isoniazid are known inhibitors of the cytoplasmic pyridoxine kinase (which converts unphosphorylated forms of vitamin B₆ into phosphorylated forms) (10, 12) and since the initial rate of pyridoxine uptake occurs without metabolic alterations, it is reasonable to conclude that intracellular phosphorylation of the transported substrate is not involved in pyridoxine uptake by colonic epithelial cells under our experimental conditions. Not only colonocytes of mouse origin, but also human colonocytes appear to have a functional pyridoxine uptake mechanism. This conclusion is based on the observation of a significant inhibition in \([3H]\)pyridoxine uptake by unlabeled pyridoxine in purified native human colonic AMV isolated from the colonic mucosa of organ donors.

Previous studies have shown the pyridoxine uptake process of a number of prokaryotic and eukaryotic systems to be sensitive to the presence of amiloride (3, 4, 22–24). Our findings in the present study show that the YAMC cells are not an exception to these findings because significant inhibition in pyridoxine uptake was observed in the presence of this diuretic agent in the incubation medium. Whether amiloride also interacts with the colonic pyridoxine uptake process in vivo is not clear and requires further investigation.

The colonic pyridoxine uptake process appears to be regulated by extracellular and intracellular factors. Extracellular pyridoxine level appears to exert a marked effect on pyridoxine uptake by YAMC cells. This conclusion is based on the observation that maintaining these cells in a growth medium in the absence of pyridoxine oversupplementation led to a significantly higher uptake in \([3H]\)pyridoxine compared with uptake by cells maintained in the presence of pyridoxine oversupplementation. Similar upregulation was seen with certain other water-soluble vitamin transport in a variety of cellular systems [i.e., folate, thiamine, biotin, and riboflavin transport (1, 18, 19, 21)]. This increase in pyridoxine uptake in the absence of pyridoxine oversupplementation appears to be mediated via transcriptional/translational mechanism(s). The latter suggestion is based on the findings that the presence of actinomycin D (a transcription inhibitor) and cyclohexamide (a translation inhibitor) inhibited the induction in pyridoxine uptake caused by switching the maintenance condition of the cells from high to low pyridoxine levels. Further studies are required to delineate the exact molecular mechanism(s) involved in this adaptive response of the pyridoxine colonic uptake process by substrate availability. The colonic pyridoxine uptake process also appeared to be under intracellular regulation. This suggestion is based on the observations of inhibition in pyridoxine uptake by inhibitors of the intracellular Ca²⁺/CaM-mediated pathway. The cellular and molecular mechanisms that mediate the Ca²⁺/CaM-mediated regulation of pyridoxine uptake by colonic epithelial cells are not known, and further studies are required to address these issues. Other intracellular regulatory pathways, like the PKA-, PKC-, and NO-mediated pathways, however, appeared to exert no regulatory effect on pyridoxine uptake. These findings are similar to those observed for the pyridoxine uptake process in renal epithelial cells but are different from the findings seen with the enterocyte Caco-2 cells, where a PKA- but not a Ca²⁺/CaM-mediated pathway was found to play a role in the regulation of pyridoxine uptake (22, 23). These findings suggest that different cells use different intracellular mechanisms to regulate pyridoxine uptake.

In summary, our results show for the first time the functional existence of a specific carrier-mediated mechanism for pyridoxine uptake by mammalian colonocytes. In addition, the results show that this uptake process is regulated by extracellular and intracellular factors.

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